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Original Article

Candidate metabolite markers of peripheral neuropathy in Chinese patients with type 2 diabetes

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Abstract: Objectives: To analyze the serum and urine metabolites present in type 2 diabetes mellitus (T2DM) patients and T2DM patients with diabetic peripheral neuropathy (DPN) and to select differentially expressed biomarkers for early diagnosis of DPN. Methods: Serum and urine metabolites from 74 T2DM patients with peripheral neuropathy and 41 without peripheral neuropathy were analyzed using gas chromatograph system with time-of-flight mass spectrometer metabolomics to detect biomarkers of peripheral neuropathy in T2DM. Results: There were increased serum triglycerides, alanine aminotransferase, and decreased C-peptide, and total cholesterol levels in T2DM patients with DPN compared to those without peripheral neuropathy. Metabolomic analysis revealed visible differences in metabolic characteristics between two groups, and overall 53 serum differential metabolites and 56 urine differential metabolites were identified with variable influence on projection (VIP) >1 and $P<0.05$. To further analyze the correlation between the identified metabolites and DPN, four serum metabolites and six urine metabolites were selected with VIP>2, and fold change (FC) >1, including serum β-alanine, caproic acid, β-alanine/L-aspartic acid, and L-arabinose/L-arabitol, and urine gluconic acid, erythritol, galactonic acid, guanidoacetic acid, cytidine, and aminoadipic acid. Furthermore, five serum biomarkers and six urine biomarkers were found to show significant changes ($P<0.05$, VIP>1, and FC>1) respectively in patients with mild, moderate, and severe DPN. In addition, we found that glyoxylate and dicarboxylate metabolism was a differential metabolic pathway not only between T2DM and DPN, but also among different degrees of DPN. The differential metabolites such as β-alanine and caproic acid are expected to be biomarkers for DPN patients, and the significant changes in glyoxylate and dicarboxylate metabolism may be related to the pathogenesis of DPN. Conclusion: There were serum and urine spectrum metabolomic differences in patients with DPN, which could serve as biomarkers for T2DM and DPN patients.

Keywords: Type 2 diabetes mellitus, diabetic peripheral neuropathy, gas chromatograph system with time-of-flight mass spectrometer, metabolomics

Introduction

Diabetic peripheral neuropathy (DPN) is a type of peripheral nerve dysfunction that is the most common complication of diabetes. Typical DPN is a chronic symmetric, disease-dependent, sensorimotor multiple neuropathy [1]. It features progressive degeneration of the peripheral nerve from the distal to the proximal end, leading to a range of sensory symptoms such as pain, weakness, and/or loss of sensation. The International Diabetes Federation stated that the total number of diabetic patients in the world reached 382 million in 2013 and may increase to 592 million by 2035 [2]. The prevalence of DPN at about 5, 10, and 20 years after being diagnosed with diabetes is about 30%, 60%, and 90%, respectively [3]. DPN is closely related to morbidity and mortality. Currently, there is no effective method to treat

DPN except strict blood glucose control. It can be seen that the quality of life of type 2 diabetes mellitus (T2DM) patients is seriously affected by peripheral neuropathy [4]. Since the pathophysiology of DPN is not fully understood, it is important to identify underlying biomarkers that lead to nerve damage and prevent nerve regeneration for successful development of therapeutic intervention [5].

Metabolomics is the high-throughput characterization of small molecule metabolites (<1500 Daltons) in biological matrices using analytical chemistry techniques [6]. It is mainly divided into two analytical techniques. One is called untargeted metabolomics analysis, which uses screening and metabolite identification to detect unknown important and relevant endogenous metabolites. The other is targeted metabolomics, a quantitative analysis of known endogenous metabolites [7]. The metabolome mainly includes small molecule metabolites such as amino acids, organic acids, fatty acids, lipids and carbohydrates. Metabolomics measures metabolic changes to understand the progression of the disease and is a rapidly evolving field in systems biology [8, 9]. It is widely used in assessing the metabolic status of diabetes-related diseases and identifying candidate biomarkers [10]. Badeau *et al.* [11] concluded that short-term rosiglitazone treatment resulted in a slight improvement in metabolism in patients with T2DM and coronary heart disease (CHD) using serum nuclear magnetic resonance (NMR) metabolomics, and changes in serum lactic acid and glutamine concentrations reflected an improved insulin sensitivity. Zhao *et al.* [12] performed metabolic profiling based on an untargeted high-resolution liquid chromatography-mass spectrometry to identify seven plasma candidate biomarkers in T2DM and non-T2DM in American Indians. Wang *et al.* [13] identified candidate metabolite markers in diabetic retinopathy by gas chromatography-time-of-flight mass spectrometry (GC-TOFMS), revealing for the first time some novel metabolites in DR, such as d-2,3-dihydroxypropanoic acid, isocitric acid, and threonic acid. Our former study also showed serum and urine differential metabolites of T2DM patients with or without nephropathy based on GC-TOFMS metabolomics [14].

In this study, serum and urine metabolism were analyzed using GC-TOFMS between T2DM patients with or without peripheral neuropathy. In addition, we evaluated serum and urine metabolites in patients with varying degrees of DPN, and explored the relationship between these biomarkers and DPN progression. Our aim was to find alternative indicators to predict the diagnosis and development of DPN.

Materials and methods

Chemicals and reagents

Standards of pyridine, anhydrous sodium sulfate, methoxyamine HCl, and fatty acid methyl ester (C7-C30) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-methyl-N-trimethylsilyl trifluoroacetamide with 1% (vol) trimethylchlorosilane, methanol, hexane, acetonitrile, chloroform, dichloromethane and acetone were from Thermo-Fisher Scientific (FairLawn, NJ, USA). Ultrapure water was produced by a Mill-Q Reference system with a LC-MS Pak filter (Millipore, Billerica, MA).

Study population

74 T2DM patients with peripheral neuropathy and 41 without peripheral neuropathy were admitted to Shuguang Hospital, Putuo Hospital and Shanghai Traditional Chinese Medicine-integrated Hospital of Shanghai University of Traditional Chinese Medicine from March 2015 to March 2016.

T2DM was diagnosed according to the diabetes diagnosis standards of American Diabetes Association (ADA) in 2010: 1) glycosylated hemoglobin (HbA1c) ≥6.5%; 2) fasting blood glucose (FPG) ≥7.0 mmol/L (no calories intake for at least eight hours was the definition of fasting); 3) 2 hours of blood glucose during oral glucose tolerance test ≥11.1 mmol/L; 4) random blood glucose ≥11.1 mmol/L, in patients with typical hyperglycemia or hyperglycemia crisis [15]. DPN was diagnosed according to the Toronto clinical scoring system (TCSS) proposed by Perkins *et al.* [16] in 2001. It takes neuroelectro-physiological examination as the gold standard for diagnozing DPN, which has high diagnostic value for DPN and clinical application value in the classification of neuropathy. The TCSS score is evaluated from neurological symptoms, neural reflexes, and sensory func-

tion, with a total score of 0-19, as shown in Table S1.

All patients included in this study had no history of hypertension or other complications, and did not use relevant treatment drugs. Patients with neuropathy caused by lumbar spine disease, cerebral infarction, and Guillain-Barré syndrome were also excluded. Patients with severe arteriovenous vascular disease, drug-induced neurotoxicity, and renal insufficiency were also excluded.

According to the grading criteria of TCSS (Table S1), DPN is absent with a score of 0 to 5, mild with a score of 6 to 8, moderate with a score of 9 to 11, and severe with a score of 12 to 19. Thus in the present study, DPN patients were further divided into three groups: mild group (n=37), moderate group (n=24), and severe group (n=13).

Ethical approval of the present study was provided by the Ethics Committee of Shanghai University of Traditional Chinese Medicine. Institutional Review Board (IRB) approval report is provided in Supporting Information. Informed consent from all participants was obtained.

GC-TOFMS analysis

All serum samples were centrifuged for 5 min at 4°C and 3,000 g. 10 μL of internal standard solution, i.e. 2-chloro-phenylalanine with the concentration 0.5 mg/mL was mixed with each 50 μL serum sample, and then with 175 μL of pre-chilled methanol chloroform. Then samples were briefly evaporated to eliminate chloroform using a CentriVap vacuum concentrator (Labconco, Kansas City, MO, USA). We obtained quality control (QC) samples by combining sera from different groups.

All urine samples were centrifuged for 5 min at 4°C and 3,000 g. 10 μL of internal standard solution was mixed with each aliquot of 75 μL urine sample, and then lyophilized with a FreeZone dryer with a stopping tray dryer (Labconco, Kansas City, MO, USA). We obtained QC samples by combining urine from different groups.

After the mixture was stored in a refrigerator at -20°C for 20 min and centrifuged at 14,000 g and 4°C for 20 min, the supernatant was then carefully transferred to an autosampler vial (Agilent Technologies, Foster City, CA, USA). Afterwards, the samples were analyzed by GC-TOFMS system (Pegasus HT, Leco Corp., St. Joseph, MO, USA) coupled to an Agilent 7890B gas chromatography (Gerstel, Muehlheim, Germany) with a Rxi-5 MS capillary column (30 m*250 μm i.d., 0.25-μm; Restek corporation, Bellefonte, PA, USA). Carrier gas flow was 1.0 ml/min using helium. The temperatures of injection interface, transfer interface, and the source temperatures were 270°C, 270°C, 220°C respectively. The electron impact ionization was used 70 eV and mass spectrometry scanning range was 50-500 m/z. The QC sample was injected at the beginning of the run to ensure system equilibrium and subsequently every 15 samples to further monitor the stability of the whole analysis.

Statistical analysis

Clinical data were expressed as medians (interquartile ranges, IQRs). Statistical analysis was performed using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA). Independent sample T-test was used to evaluate the significance of differences between the two groups. One-way analysis of variance (ANOVA) was carried out for comparison of variance of the variables among the 3 groups. A difference with a P value <0.05 was considered significant.

The raw data generated by GC-TOFMS analysis were processed using XploreMET (MetaboProfile, Shanghai, China) for automated baseline denoizing and smoothing, peak picking and deconvolution, creating a reference database from the pooled QC samples, metabolite signal alignment, missing value correction and imputation, and QC correction. Categorical variables were compared by Chi-square test. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using SIMCA-P12.0 (Umetrics AB). The metabolites with P (adjusted by false discovery rate (FDR) method proposed by Benjamini and Hochburg [17]) <0.05, variable influence on projection (VIP) >1 and fold change (FC) value >1 were considered as candidate biomarkers for classification. Then, the differentially metabolites were presented in volcano plot. Data in Supplementary Materials are shown as medians (IQRs).

The differential compounds were matched to Human Metabolome Database (HMDB, http://www.hmdb.ca/) to identify putative differential metabolites. Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) pathway enrichment analysis was performed using the differentially expressed metabolites, and *P* value <0.05 was considered significantly enriched. Biomarker analysis of differentially expressed metabolites was conducted at MetaboAnalyst 3.0 web server (http://www.metaboanalyst.ca).

The diagnostic performance of biomarkers in serum and urine samples was visualized by receiver operating characteristic (ROC) curves. For the sensitivity, specificity, and predictive values were calculated at 95% confidence intervals (CI) and the area under the curve (AUC).

Results

Clinical characteristics of T2DM patients with and without peripheral neuropathy

Table 1 summarizes the clinical characteristics of 120 subjects, including 74 T2DM patients with and 41 without peripheral neuropathy. As shown in **Table 1**, there were no significant differences in the age, body mass index (BMI), or gender ratio between the two groups. In the T2DM patients with peripheral neuropathy group, waist-to-hip ratio, triglyceride, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) level were distinctly increased more than those without peripheral neuropathy ($P<0.05$), while C-peptide, insulin and total cholesterol (TC) were lower than those in T2DM patients without peripheral neuropathy ($P<0.05$). In addition, accompanied by the severity of DPN, serum TC level gradually decreased. The detailed clinical features of T2DM patients with or without peripheral neuropathy are shown in Table S2.

GC-TOFMS data analysis of serum and urine in T2DM patients with or without peripheral neuropathy

A total of 183 serum metabolites including amino acids, nucleotide, carbohydrates, fatty acids, organic acid, and others were identified. PCA was originally used to summarize GC-TOFMS data and exclude any outliers. From the PCA score plots for serum, it can be seen that most samples were clustered in the 95% confidence interval (the oval area of the score chart). According to the PCA score plots of serum, the clustering trend of the T2DM and DPN groups was not obvious (Figure S1A). To further distinguish the differences between the two groups, OPLS-DA analysis was performed (**Figure 1A**). The results showed that the established model was effective with good pattern quality. As shown in **Figure 1A**, the T2DM and DPN groups can be distinguished by serum OPLS-DA scores, and the clustering trend of the two groups was obvious. The values of R2X, R2Y, and Q2 of the two groups of OPLS-DA models were 0.277, 0.895, and 0.778, respectively, which suggested that the established model was reliable. The results of 1000 permutation tests showed that the Q2 intercept of serum model was -0.43, which was a negative value, indicating that the model was effective (**Figure 1B**).

We identified 161 urine metabolites including amino acids, nucleotides, carbohydrates, organic acids, vitamin and others. From the PCA score plots for urine, it can be seen that most samples were clustered in the 95% confidence interval (Figure S1B). To further distinguish the differences between the two groups, OPLS-DA analysis was performed (**Figure 1C**). As shown in **Figure 1C**, the T2DM and DPN groups can be distinguished, and a clustering trend of the two groups was obvious. The values of R2X, R2Y, and Q2 of the two groups of OPLS-DA models were 0.170, 0.821, and 0.628, respectively, which suggested that the established model was reliable. The results of 1000 permutation tests showed that the Q2 intercept of urine model was -0.393, which was a negative value, indicating that the model was effective (**Figure 1D**).

Metabolic characters in serum and urine of T2DM patients with or without peripheral neuropathy

Combined with PCA and OPLS-DA analysis, differential serum metabolites were also selected using univariate statistical analysis (Figure S2A). Differential metabolites are shown as an enhanced volcano plot (V-plot) (Figure S2B). The *P* value and log 1.5 FC were calculated, with cut-off values of 0.05, *P* values of 0.01,

Table 1. Clinical information of T2DM patients with and without peripheral neuropathy

Characteristic	T2DM (n=41)	DPN (n=74)			
		Overall	DPN-Mild (n=37)	DPN-Moderate (n=24)	DPN-Severe (n=13)
Gender (Male/Female)	29/12	46/28	19/18	19/5	8/5
Age (years)	71 (55-79)	64 (58.75-74)	63 (55.5-71)	66 (60-76)	62 (60-79)
Body mass index (kg/m^2)	24.34 (22.86-26.12)	24.49 (23.2-27.04)	24.04 (22.49-26.42)	24.57 (23.51-26.69)	26.2 (24.49-28.13)
Waist-to-hip ratio	0.93 (0.91-0.96)	0.95 (0.93-0.98)*	0.95 (0.9-0.97)	0.94 (0.94-0.99)	0.95 (0.95-0.99)
Glucose (mmol/L)	7.5 (5-9.6)	7.6 (5.57-9.55)	7.42 (5.24-9.03)	7.2 (6.02-8.86)	8.97 (6.46-10.6)
Two hours post-load plasma glucose (mmol/L)	11 (6.98-14.53)	11.54 (8.27-14.27)	13.46 (9.19-14.8)	9.86 (8.02-13)	8.41 (6.89-12.1)
C-peptide (nmol/L)	1.41 (0.88-2.01)	0.71 (0.43-1.01)***	0.9 (0.48-1.12)	0.63 (0.43-0.8)	0.46 (0.35-0.67)
Two hours C-peptide (nmol/L)	1.96 (1.07-4.01)	1.34 (0.8-2.75)	1.55 (0.94-3.12)	1.38 (0.94-2.45)	0.8 (0.51-1.5)
Insulin (mmol/L)	69.74 (44.59-107.73)	12.36 (5.56-40.12)***	11.15 (5.56-337.2)	11.8 (6.8-26.41)	37.52 (23.1-52.93)
HbA1c (%)	8.4 (6.98-9.58)	8.5 (7.05-10)	8.5 (7.2-10.25)	8.1 (7-8.9)	9.25 (7.53-10.25)
ALT (U/L)	14 (111-22)	19.5 (15-27.75)**	20 (16.2-27)	18.1 (15-25.45)	20 (12-32)
AST (U/L)	17 (13-21)	20.5 (16-25)**	20 (16-28)	21.5 (16.75-24.5)	20 (16-22)
Triglyceride (mmol/L)	1.05 (0.85-1.37)	3.86 (1.52-4.86)***	3.81 (1.49-5.06)	3.57 (1.49-4.57)	4.04 (3.3-4.75)
TC (mmol/L)	3.92 (3.53-5.09)	2.6 (1.55-4.21)***	3.48 (2.01-4.51)	2.75 (1.4-4.03)	1.36 (0.99-1.84)##
High-density lipoprotein (mmol/L)	1.01 (0.84-1.17)	1 (0.85-1.13)	0.91 (0.83-1.06)	1.04 (0.9-1.21)	1.08 (0.88-1.16)
Low-density lipoprotein (mmol/L)	2.52 (2.06-3.29)	2.56 (2.05-3.12)	2.65 (2.23-3.21)	2.47 (1.76-3.11)	2.64 (2.06-2.92)

Note: Data were expressed as the medians (IQRs). The Wilcoxon test was used to compare these variables between T2DM patients with and without peripheral neuropathy, and $P<0.05$ was significant. ANOVA was used to compare these variables among the three degrees of DPN. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, Comparison of T2DM patients and DPN patients; ##, $P<0.01$, Comparison of severe DPN patients with moderate DPN patients. T2DM, Type 2 diabetes mellitus; ANOVA, Wilcoxon test and analysis of variance; DPN, Diabetic peripheral neuropathy.

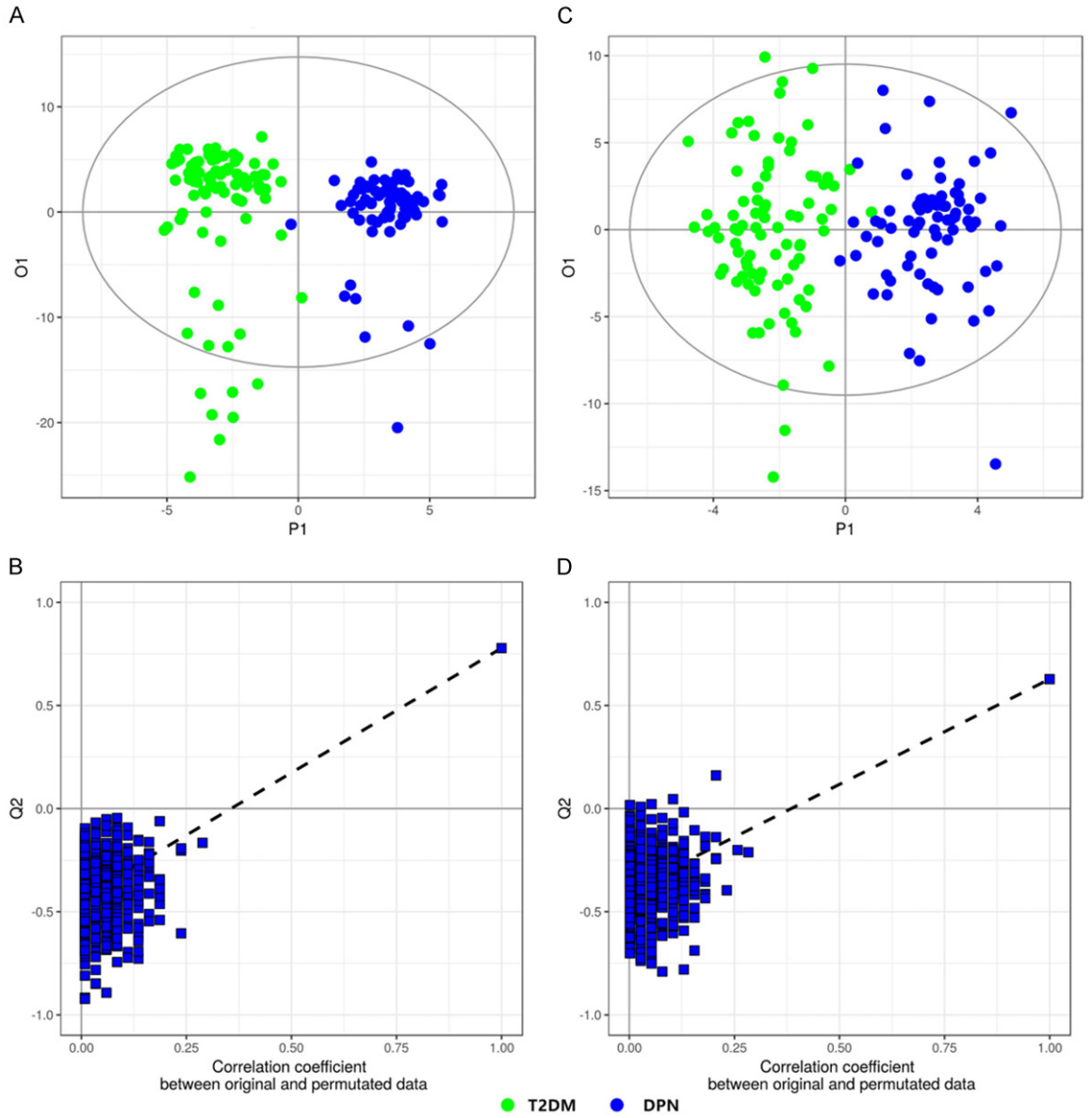

Figure 1. OPLS-DA score plot of T2DM patients with or without peripheral neuropathy. A. OPLS-DA score plot of serum metabolites. B. 1000 permutation test plot of serum metabolites. C. OPLS-DA score plot of urine metabolites. D. 1000 permutation test plot of urine metabolites. OPLS-DA, Orthogonal partial least squares-discriminant analysis; T2DM, Type 2 diabetes mellitus; DPN, Diabetic peripheral neuropathy.

and 1.5 for log 1.5 FC. The metabolites and metabolic pathways were further identified on the basis of accurate molecular weight and mass spectra, and verified using HMDB and KEGG databases. The information of 53 candidate metabolite markers with VIP>1 and *P* value <0.05 is provided in **Table 2**. Additional information is provided in Table S3. To further analyze the correlation between the identified metabolites and DPN, 4 metabolites were selected with *P*<0.05, VIP>2, and FC>1. Among them, **Figure 2A** shows the 4 most significant changes in serum metabolites. Serum β-alanine, ratio of β-alanine/L-aspartic acid, caproic acid and ratio of L-arabinose/L-arabitol in DPN patients was significantly increased compared to T2DM patients. To determine which serum metabolic pathways are primarily relevant for DPN development, we performed pathway analysis according to their *P* values in their enrichment and impact values. As shown in Figure S3A, the "metabolomics view" demon-

Table 2. Differential serum metabolites between T2DM patients with and without peripheral neuropathy

Class	Name	*P*	VIP	FC
Amino Acid	β-Alanine	2.78E-13	3.4	10.3
	Ratio of β-Alanine/L-Aspartic acid	6.69E-12	3.3	8.3
	Ratio of Urea/L-Arginine	2.55E-03	1.5	2.2
	Ketoleucine	2.30E-03	1.3	1.3
	L-Alpha-aminobutyric acid	3.91E-03	1.3	1.3
	Ratio of Ketoleucine/L-Leucine	6.23E-03	1.2	1.3
	Ratio of L-Serine/Glycine	8.55E-03	1.2	1.2
	L-Glutamine	2.51E-04	1.6	0.9
	L-Threonine	1.34E-02	1.3	0.9
	Ratio of Glycine/L-Serine	6.48E-03	1.2	0.9
	Ornithine	1.91E-02	1.2	0.9
	Dimethylglycine	3.21E-02	1.2	0.9
	Glycine	3.52E-03	1.7	0.8
	Ratio of L-Glutamic acid/Pyroglutamic acid	5.98E-03	1.2	0.8
	D-2-Hydroxyglutaric acid	6.17E-03	1.2	0.8
	Ratio of L-Glutamic acid/Oxoglutaric acid	8.44E-03	1.1	0.8
	4-Hydroxyproline	1.47E-02	1.1	0.8
	L-Asparagine	2.42E-08	2.5	0.7
	Citrulline	9.73E-04	1.5	0.7
	L-Kynurenine	1.95E-03	1.4	0.7
	Ratio of L-Asparagine/L-Aspartic acid	9.87E-04	1.3	0.7
	1-Methylhistidine	9.02E-03	1.2	0.7
	L-Arginine	2.53E-06	2	0.6
	N-Acetyl-L-aspartic acid	4.40E-03	1.1	0.6
	Homocysteine	4.12E-09	2.4	0.5
Carbohydrates	Ratio of L-Arabinose/L-Arabitol	8.12E-07	2.3	1.5
	Allose	3.67E-03	1.3	0.9
	D-Galactose	2.16E-03	1.7	0.8
	D-Mannose	1.05E-02	1.2	0.8
	Erythritol	1.09E-02	1.1	0.8
	D-Threitol	1.10E-02	1.1	0.8
	Threonic acid	1.33E-02	1.1	0.8
	L-Arabitol	6.11E-06	2.1	0.7
	D-Xylose	3.26E-06	2	0.7
	Rhamnose	2.96E-04	1.6	0.7
	Isomaltose	1.82E-03	1.4	0.6
	D-Maltose	4.04E-03	1.3	0.6
	Ribitol	9.74E-03	1.4	0.4
Fatty Acids	Caproic acid	3.46E-10	3	1.9
	Arachidic acid	1.49E-04	1.6	1.5
	Pelargonic acid	9.61E-05	1.8	1.3
	Docosahexaenoic acid	7.34E-04	1.4	1.2
Organic Acids	L-Pipecolic acid	3.15E-02	1.1	0.8
	Succinic acid	5.54E-10	2.9	0.7
	Glycolic acid	7.71E-04	1.6	0.7
Nucleotide	Ratio of Uridine/Cytidine	3.70E-03	1.1	1.6
	Cytidine	2.84E-03	1.3	0.6

Alcohols	2-Hydroxypyridine	7.59E-04	1.6	0.9
	Myoinositol	2.25E-03	1.5	0.9
	Glycerol	1.38E-03	1.5	0.8
Lipids	Cholesterol	2.94E-02	1.1	1
	O-Phosphoethanolamine	5.94E-05	1.8	0.7
Phosphate	Phosphate	8.27E-05	1.9	0.7

Note: Differential metabolites were selected according to VIP>1 and $P<0.05$. P values were calculated by t test for continuous variables and adjusted by the FDR method. T2DM, Type 2 diabetes mellitus; VIP, Variable influence on projection; FC, Fold change; FDR, False discovery rate.

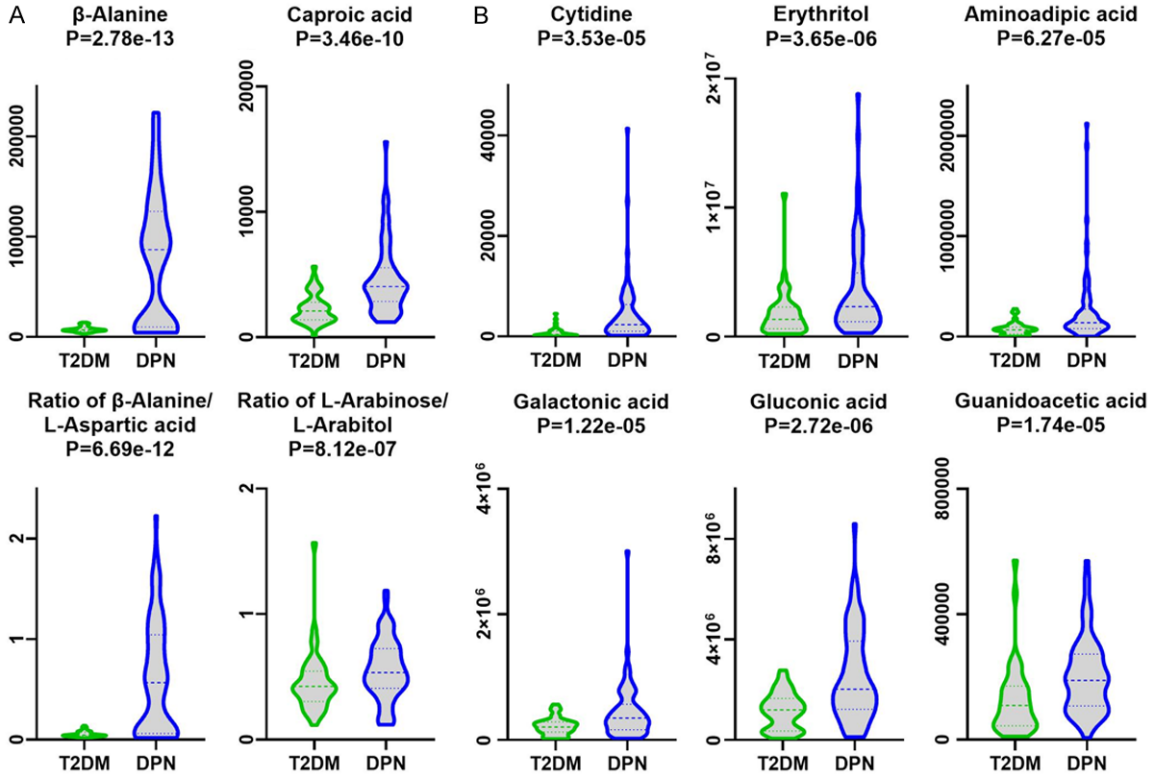

Figure 2. Representative differential metabolites between T2DM patients with or without peripheral neuropathy. A. The peak height comparison of representative serum metabolites between two groups, T2DM (green), DPN (blue), $P<0.05$, VIP value >2, and FC>1. The statistical analysis of the significance between T2DM patients with or without peripheral neuropathy. β-Alanine, $P=2.78E-13$; ratio of β-Alanine/L-Aspartic acid, $P=6.69E-12$; caproic acid, $P=3.46E-10$; ratio of L-Arabinose/L-Arabitol, $P=8.12E-07$. B. The peak height comparison of representative urinary metabolites between two groups, T2DM (green), DPN (blue), $P<0.05$, VIP value >2 and FC>1. The statistical analysis of the significance between T2DM patients with and without peripheral neuropathy group. Gluconic acid, $P=2.72E-06$; Erythritol, $P=3.65E-06$; Galactonic acid, $P=1.22E-05$; Guanidoacetic acid, $P=1.74E-05$; Cytidine, $P=3.53E-05$. T2DM, Type 2 diabetes mellitus; DPN, Diabetic peripheral neuropathy; VIP, Variable influence on projection; FC, Fold change.

strates all metabolic pathways, arranged based on the scores of enrichment analysis (Y-axis) and topological analysis (X-axis), with the most distinct P values shown in red and the least distinct P values shown in yellow and white. MetaboAnalyst 3.0 was used to perform functional enrichment analysis to observe the biological significance of metabolic changes [18], and metabolite set enrichment analysis (MSEA) on serum metabolites. Finally, there were 6 serum metabolic pathways (P value <0.05) with significant differences respectively (**Figure 3A**, **Table 4**). The occurrence of DPN serum metabolism was closely related to 6 metabolic pathways, including arginine biosynthesis, alanine, aspartate and glutamate metabolism, galac-

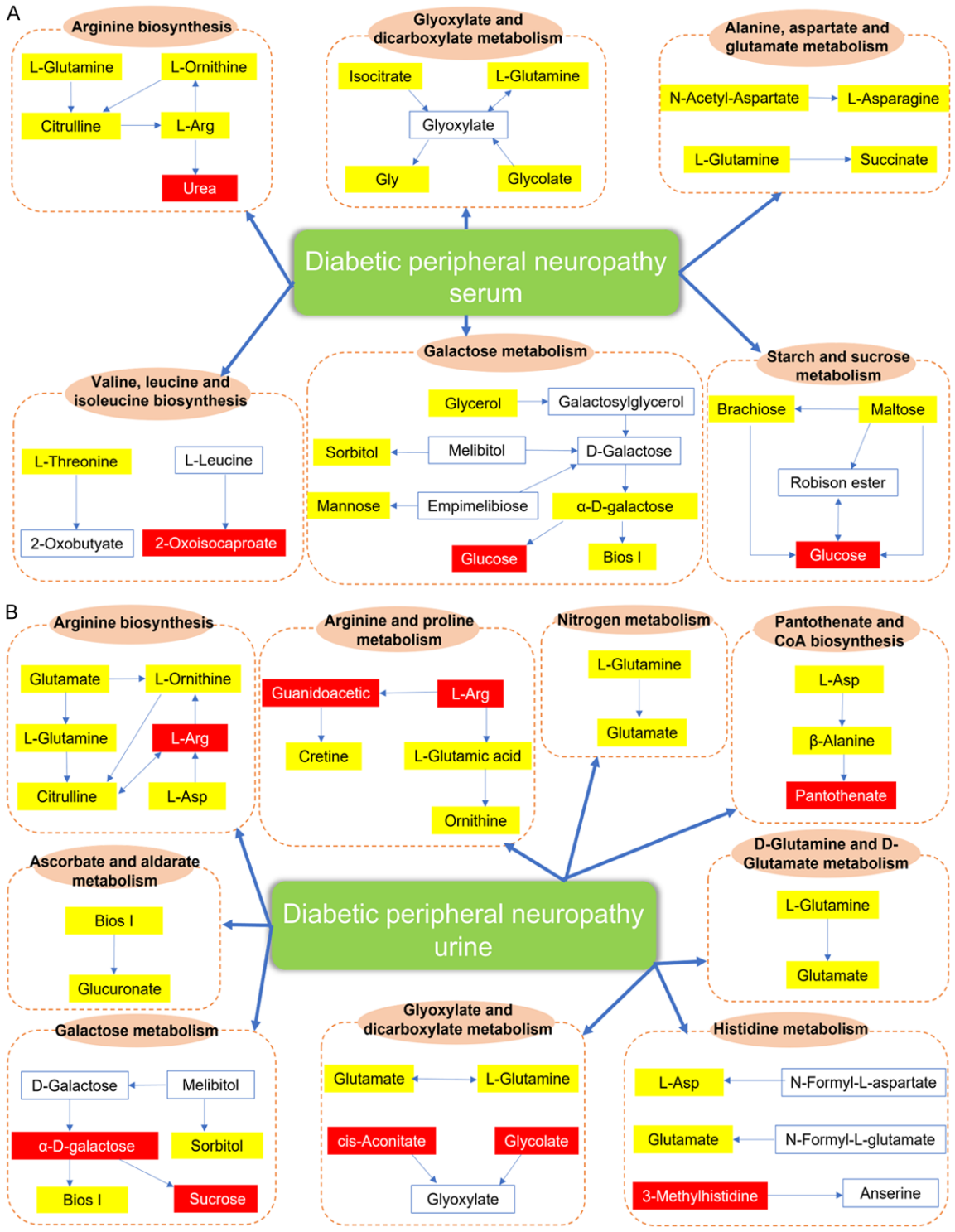

Figure 3. Representative differential metabolic pathways between T2DM patients with or without peripheral neuropathy. A. The representative metabolic pathways of serum metabolic pathways. B. The representative metabolic pathways of urine metabolic pathways. The up-regulated or down-regulated metabolites are highlighted with red and yellow respectively. T2DM, type 2 diabetes mellitus; L-Asp, L-aspartic acid; L-Arg, L-arginine; Gly, Glyceric acid.

tose metabolism, starch and sucrose metabolism, valine, leucine and isoleucine biosynthesis, glyoxylate and dicarboxylate metabolism.

At the same time, we also used univariate statistical analysis to screen for differential urine metabolites (Figure S2C). Differential metabo-

Table 3. Differential urinary metabolites in T2DM patients with and without peripheral neuropathy

Class	Name	*P*	VIP	FC
Amino Acid	Guanidoacetic acid	1.74E-05	2.4	1.7
	L-Kynurenine	2.26E-04	2.1	0.6
	Aminoadipic acid	6.27E-05	2	2.1
	L-Glutamine	2.10E-03	2	0.7
	Ratio of Citrulline/L-Arginine	1.69E-03	2	0.4
	L-Methionine	4.75E-04	1.9	0.7
	3-Nitrotyrosine	9.05E-04	1.8	1.9
	Ratio of Guanidoacetic acid/Glycine	6.62E-03	1.8	1.8
	Citrulline	4.68E-03	1.7	0.8
	1-Methylhistidine	9.72E-05	1.5	1.7
	Ratio of Creatine/Guanidoacetic acid	2.84E-03	1.5	0.4
	Ratio of L-Aspartic acid/N-Acetyl-L-aspartic acid	2.68E-03	1.4	0.7
	L-Aspartic acid	9.86E-03	1.4	0.7
	4-Hydroxy-L-proline	4.43E-03	1.3	0.8
	Ketoleucine	5.88E-03	1.3	0.6
	β-Alanine	4.02E-02	1.2	0.9
	Creatine	1.00E-02	1.2	0.7
	L-Glutamic acid	4.39E-02	1.1	0.9
	Ratio of L-Glutamine/L-Glutamic acid	4.41E-02	1.1	0.8
	Ratio of L-Serine/Glycine	4.57E-02	1.1	0.8
Carbohydrates	Gluconic acid	2.72E-06	2.8	1.7
	Galactonic acid	1.22E-05	2.6	1.9
	Erythritol	3.65E-06	2.5	2.5
	D-Glucuronic acid	9.72E-05	2.3	0.7
	Sorbitol	1.30E-04	1.9	0.7
	D-Ribose	1.25E-04	1.8	1.3
	Ribitol	3.40E-03	1.7	1.5
	Rhamnose	3.34E-04	1.6	1.8
	D-Galactose	1.39E-02	1.6	1.5
	Ratio of Gluconic acid/Gluconolactone	1.49E-02	1.5	2.5
	Ribonolactone	1.06E-02	1.4	1.3
	Mannitol	4.66E-02	1.3	0.7
	D-Xylose	1.64E-03	1.2	1.4
	Ratio of L-Arabinose/L-Arabitol	3.64E-02	1.2	1.1
	Sucrose	4.18E-02	1.1	1.7
	D-Threitol	4.37E-02	1.1	1.2
Organic Acids	Oxalic acid	2.25E-02	1.6	1.6
	p-Hydroxyphenylacetic acid	1.49E-02	1.6	0.9
	Malic acid	2.15E-03	1.6	0.5
	Tartaric acid	7.27E-03	1.4	0.3
	Suberic acid	2.32E-02	1.3	0.7
	Guanidinosuccinic acid	3.89E-03	1.3	0.6
	Glycolic acid	3.66E-02	1.2	1.4
	Uric acid	6.12E-03	1.2	1
	Hydroxyphenyllactic acid	3.97E-02	1.2	0.8
	Pimelic acid	4.64E-02	1.1	0.8
	Vanillic acid	1.47E-02	1.1	0.7

Nucleotide	Cytidine	3.53E-05	2.3	3.1
	Ratio of Uridine/Cytidine	6.34E-04	1.7	0.3
	Ratio of Hypoxanthine/Inosine	1.14E-02	1.2	1.6
	Ratio of Xanthine/Xanthosine	4.15E-02	1.1	1
Alcohols	Myoinositol	1.65E-02	1.1	0.7
Lipids	O-Phosphoethanolamine	3.43E-02	1.2	1
Vitamin	Pantothenic acid	1.94E-04	1.9	1.7
Hormone	Normetanephrine	4.56E-02	1.2	0.7
Indoles	Indoxyl sulfate	6.65E-03	1.2	0.6

Note: Differential metabolites were selected according to VIP>1 and $P<0.05$. P values were calculated from t test for continuous variables and adjusted by the FDR method. T2DM, Type 2 diabetes mellitus; VIP, Variable influence on projection; FC, Fold change; FDR, False discovery rate.

Table 4. Serum and urine metabolic pathways that significantly changed between T2DM patients with and without peripheral neuropathy

Sample	Pathway name	*P*	Impact	Up	Down
Serum	Arginine biosynthesis	2.26E-04	0.38	Urea	Ornithine; L-Arginine; L-Glutamine; Citrulline
	Galactose metabolism	9.00E-04	0.03	D-Glucose	Glycerol; D-Galactose; D-Mannose; Myoinositol; Sorbitol
	Alanine, aspartate and glutamate metabolism	3.33E-02	0.23		L-Asparagine; Succinic acid; L-Glutamine; N-Acetyl-L-aspartic acid
	Starch and sucrose metabolism	4.31E-02	0.12	D-Glucose	D-Maltose; Isomaltose
	Glyoxylate and dicarboxylate metabolism	4.63E-02	0.12		Glycolic acid; Glycine; Isocitric acid; L-Glutamine
	Valine, leucine and isoleucine biosynthesis	4.64E-02	0.25	Ketoleucine	L-Threonine
Urine	Arginine biosynthesis	1.32E-05	0.56	L-Arginine	L-Glutamic acid; L-Aspartic acid; Ornithine; L-Glutamine; Citrulline
	D-Glutamine and D-Glutamate metabolism	1.75E-02	0.67		L-Glutamic acid; L-Glutamine
	Arginine and proline metabolism	2.31E-02	0.22	Guanidoacetic acid; L-Arginine	Creatine; L-Glutamic acid; Ornithine
	Nitrogen metabolism	2.56E-02	0.25		L-Glutamic acid; L-Glutamine
	Galactose metabolism	2.81E-02	0.09	D-Galactose; Sucrose	Myoinositol; Sorbitol
	Histidine metabolism	3.03E-02	0.13	1-Methylhistidine	L-Glutamic acid; L-Aspartic acid
	Glyoxylate and dicarboxylate metabolism	4.41E-02	0.16	cis-Aconitic acid; Glycolic acid	L-Glutamic acid; L-Glutamine
	Ascorbate and aldarate metabolism	4.51E-02	0.2		D-Glucuronic acid; Myoinositol
	Pantothenate and CoA biosynthesis	4.78E-02	0.11	Pantothenic acid	β-Alanine; L-Aspartic acid

Note: Differential metabolic pathways were selected according to $P<0.05$. T2DM, Type 2 diabetes mellitus.

lites are shown in enhanced V-plot (Figure S2D). The information of 56 candidate metabolite marker with VIP>1 and P value <0.05 is provided in **Table 3**. Additional information is provided in Table S4. The 6 most significant urinary metabolites are shown in **Figure 2B** ($P<0.05$, VIP>2, and FC>1). Urine levels of gluconic acid, erythritol, galactonic acid, guanidoacetic acid, cytidine, aminoadipic acid were observably increased in the DPN group compared to T2DM patients. To determine which urine metabolic pathways are primarily relevant for DPN development, we performed pathway analysis according to their P values in their enrichment and impact values. Figure S3B presented a "metabolomics view" of all meta-

bolic pathways. As shown in **Table 4** and **Figure 3B**, 9 urinary metabolic pathways were associated with the occurrence of DPN, including arginine biosynthesis, arginine and proline metabolism, D-glutamine and D-glutamate metabolism, nitrogen metabolism, histidine metabolism, galactose metabolism, glyoxylate and dicarboxylate metabolism, ascorbate and aldarate metabolism, and pantothenate and CoA biosynthesis.

Predictive abilities of DPN-related biomarkers

It is well-known that untargeted metabolomics data have a wide variety of compound classes, massive data files, complex compound structures, and a lack of databases for searching possible compounds [19]. These factors make it difficult to screen for useful biomarkers and analyze metabolic pathways. In this study, to select the metabolites as candidates that may participate in DPN pathophysiology, compounds with significant changes between groups (*P* value <0.05, VIP>2 and FC>1) were selected as biomarkers. Four and six metabolites with the most significant changes were found in serum and urine samples from patients with DPN, respectively. Serum metabolites were β-alanine, ratio of β-alanine/L-aspartic acid, caproic acid, and ratio of L-arabinose/L-arabitol (**Figure 2A**). Urine metabolites were gluconic acid, erythritol, galactonic acid, guanidoacetic acid, cytidine, and aminoadipic acid (**Figure 2B**). We then performed ROC analysis of serum metabolites and urine metabolites separately to evaluate the clinical potential of these metabolites to predict DPN in T2DM patients. As shown in Figure S4 and Table S5, all four metabolites in serum samples showed good accuracy with high AUC values (AUC=0.946) for the prediction of DPN in T2DM patients. In urine samples, AUC value for these six metabolites were also high, at 0.946 for predicting DPN in T2DM patients.

Serum and urinary metabolic profiles in T2DM patients with peripheral neuropathy of different degrees

We further analyzed the serum metabolites of the three groups with different degrees of DPN. PCA was utilized to show similarities and differences among the mild, moderate, and severe groups of DPN patients in serum metabolites (Figure S5A). For the OPLS-DA model, the score of serum in mild, moderate, and severe groups could easily distinguish the three groups (**Figure 4A-F**). The values of R2X, R2Y, and Q2 of the three groups of OPLS-DA models were 0.252, 0.444, and -0.0965, respectively. 5 candidate serum biomarkers were finally identified (*P*<0.05, VIP>1, and FC>1), including β-alanine, hydroxylamine, putrescine, L-kynurenine, and ratio of β-alanine/L-aspartic acid (**Figure 5A**). Details are shown in **Table 5**, and additional information is provided in Table S6. So as to ascertain which serum metabolic pathways were most affected in different degrees of DPN, we analyzed these pathways based on the enrichment and impact values of *P* values. As shown in Figure S6A-C, the "metabolome view", which demonstrates all metabolic pathways, was arranged with the most significant *P* values. As shown in **Table 6** and **Figure 6A**, the occurrence of DPN serum metabolism was closely related to 10 metabolic pathways, including arginine biosynthesis, D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, nitrogen metabolism, histidine metabolism, glyoxylate and dicarboxylate metabolism, galactose metabolism, starch and sucrose metabolism, valine, leucine and isoleucine biosynthesis, and cysteine and methionine metabolism.

We also analyzed the urine metabolites of the three groups with different degrees of DPN. PCA was utilized to show similarities and differences among the mild, moderate, and severe groups of DPN patients in urine metabolites (Figure S5B). For the OPLS-DA model, the score of urine in mild, moderate and severe groups could easily distinguish the three groups (**Figure 4G-L**). The values of R2X, R2Y, and Q2 of the three groups of OPLS-DA models were 0.199, 0.569, and -0.0839, respectively. 6 candidate urine biomarkers were finally identified (*P*<0.05, VIP>1, and FC>1), including L-arabinose, D-2-hydroxyglutaric acid, adipic acid, cis-aconitic acid, D-xylose, and glycine (**Figure 5B**, **Table 5** and Table S6). So as to ascertain which urine metabolic pathways were most affected in different degrees of DPN, we analyzed these pathways based on the enrichment and impact values of *P* values (Figure S6D-F). As shown in **Table 6** and **Figure 6B**, 7 urinary metabolic pathways were closely associated with the occurrence of DPN, including

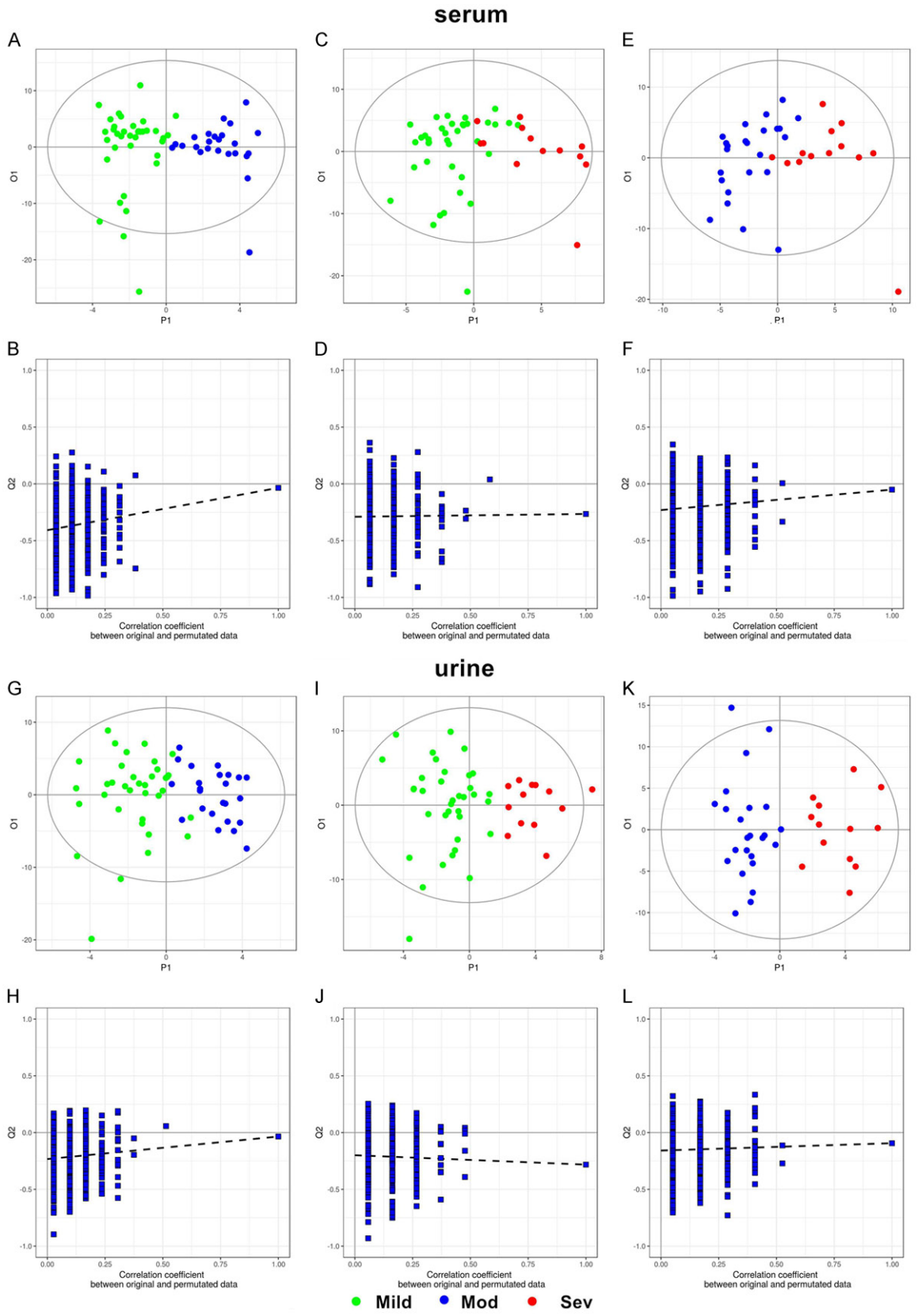

Figure 4. OPLS-DA score plots for metabolic profiling in different degrees of DPN. A. OPLS-DA score plot of serum metabolites between mild group and moderate group. B. 1000 permutation test plot. C. OPLS-DA score plot of serum

metabolites between mild group and severe group. D. 1000 permutation test plot. E. OPLS-DA score plot of serum metabolites between moderate group and severe group. F. 1000 permutation test plot. G. OPLS-DA score plot of urinary metabolites between mild group and moderate group. H. 1000 permutation test plot. I. OPLS-DA score plot of urinary metabolites between mild group and severe group. J. 1000 permutation test plot. K. OPLS-DA score plot of urinary metabolites between moderate group and severe group. L. 1000 permutation test plot. OPLS-DA, Orthogonal partial least squares-discriminant analysis; DPN, Diabetic peripheral neuropathy.

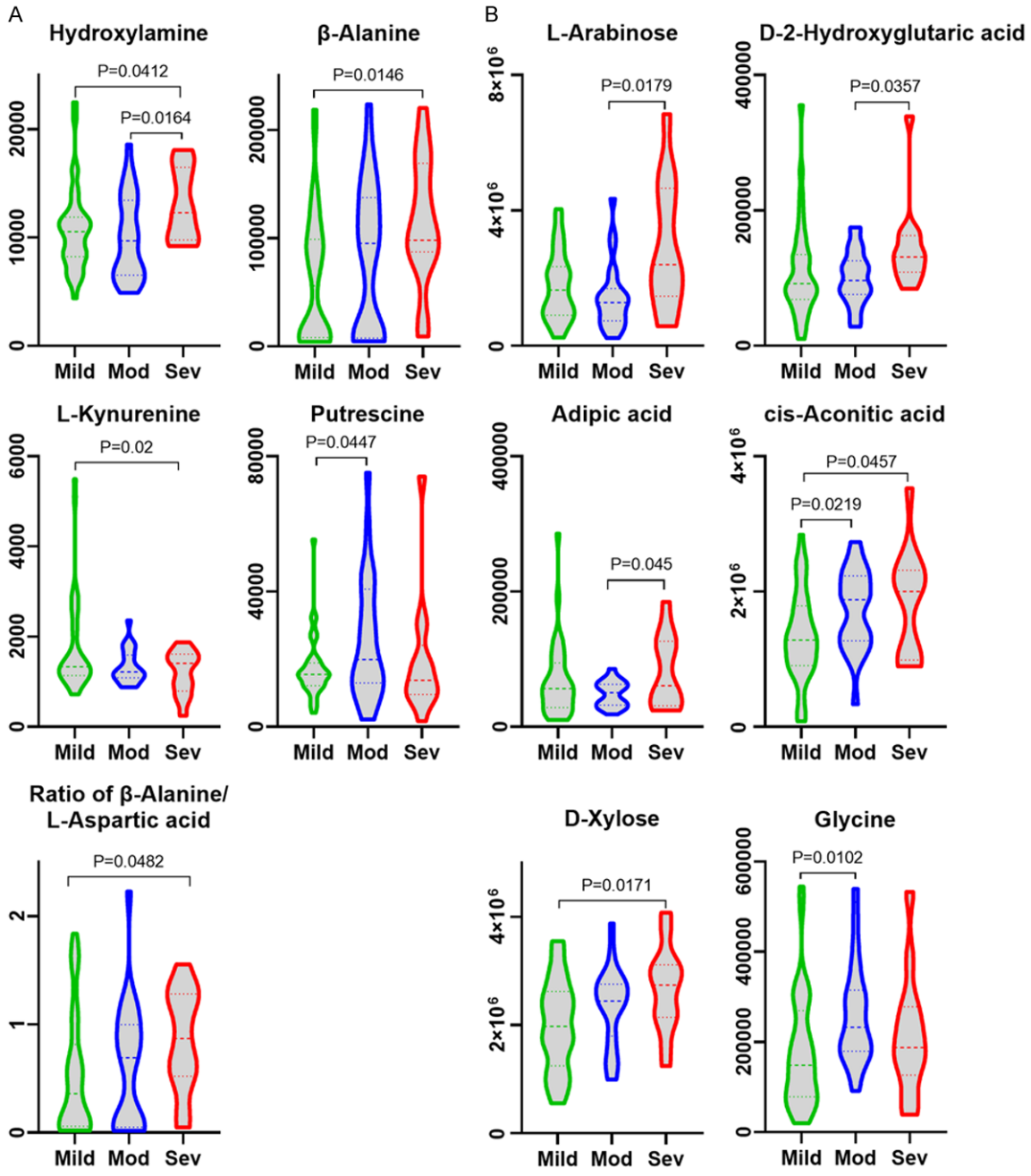

Figure 5. Representative differential metabolites in different degrees of DPN. A. Pairwise comparison of peak heights of three representative serum metabolites, Mild (green), Moderate (blue), Severe (red), $P<0.05$, VIP>1, and FC>1. B. Pairwise comparison of peak heights of three representative urinary metabolites, Mild (green), Moderate (blue), Severe (red), $P<0.05$, VIP>1, and FC>1. DPN, Diabetic peripheral neuropathy; VIP, Variable influence on projection; FC, Fold change.

Table 5. Serum and urine metabolites that significantly changed among DPN mild, moderate, and severe groups

Sample	Class	Name	Mod-Mild			Sev-Mild			Sev-Mod		
			P	VIP	FC	*P*	VIP	FC	*P*	VIP	FC
Serum	Amino Acids	β-Alanine	0.221	0.7	1.7	1.46E-02	1.8	1.8	0.17	1.2	1
		Ratio of β-Alanine/L-Aspartic acid	0.534	0.1	1.9	4.82E-02	1.4	2.4	0.179	1.2	1.3
		L-Kynurenine	4.12E-02	1.5	0.9	2.00E-02	1.6	1.1	0.399	0.8	1.2
	Alkylamines	Hydroxylamine	0.498	0.2	0.9	4.12E-02	1.5	1.2	1.64E-02	2.1	1.3
		Putrescine	4.47E-02	2.7	1.3	0.663	0.4	0.9	0.345	1.1	0.7
Urine	Amino Acids	D-2-Hydroxyglutaric acid	0.505	0.2	1.1	0.112	2	1.4	3.57E-02	2.9	1.4
		Glycine	1.02E-02	1.9	1.6	0.386	1.4	1.3	0.319	0.8	0.8
	Carbohydrates	L-Arabinose	0.228	0.9	0.8	0.052	2.2	1.5	1.79E-02	2.8	1.9
		D-Xylose	0.099	1.3	1.2	1.71E-02	2	1.4	0.23	0.9	1.1
	Organic Acids	Adipic acid	0.054	1.4	0.9	0.426	0.6	1.1	4.50E-02	2.6	1.2
		cis-Aconitic acid	2.19E-02	2.5	1.5	4.57E-02	2.3	1.6	0.583	0.4	1.1

Note: Differential metabolites were selected according to $P<0.05$, VIP>1, and FC>1. ANOVA was conducted in the comparison among DPN different degrees. T-test was performed in the comparison between two groups. DPN, Diabetic peripheral neuropathy; VIP, Variable influence on projection; FC, Fold change; ANOVA, Wilcoxon test and analysis of variance.

glyoxylate and dicarboxylate metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis, pentose and glucuronate interconversions, ascorbate and aldarate metabolism, β-alanine metabolism, and arginine biosynthesis.

Discussion

T2DM is a disorder of glucose and lipid metabolism. Studies show that among adults aged 20-79, the prevalence of diabetes is estimated to be 8.8% by 2015, and will rise to 10.4% by 2040 [20]. In recent years, the incidence of T2DM and its complications has also increased accompanied by changes in people's lifestyle in China [21]. DPN is a common complication of T2DM, and is found in 50% patients with T2DM [22]. About 11% of patients with DPN have chronic pain symptoms, which severely decreases the quality of life of T2DM patients and is one of the main reasons for the increased incidence and mortality of DPN [23]. According to epidemiologic studies, hypertension and obesity, high blood glucose and glycated hemoglobin levels, duration of diabetes, and increased albumin excretion rates are major risk factors for DPN [24]. However, the pathological progress of DPN is still unclear. Thus, it is important to identify biomarkers in T2DM for better diagnosis of DPN.

In our study, as shown in **Table 1**, the serum levels of C-peptide in DPN patients were significantly decreased compared to T2DM patients. C-peptide is a hormone-active peptide [25]. Qiao *et al.* [26] recruited 220 patients with T2DM, assessed DPN by clinical symptoms, signs, and electromyography, and found that C-peptide was negatively correlated with DPN in patients with T2DM. Wahren *et al.* [27] showed that C-peptide can improve vibration perception threshold compared with the placebo group. These results show that C-peptide replacement therapy can improve the neurological function of patients with early symptoms of diabetic neuropathy, and also provide a basis for the reduction of serum C-peptide in patients with DPN. In addition, we also noticed that serum TC levels in DPN patients were significantly reduced compared with T2DM patients. Along with the severity of DPN, serum TC level gradually decreased. Our finding was consistent with that of Xu *et al*. [28] which showed that TC was one significant risk determinant for DPN in patients with T2DM. However, the relationship between TC and DPN was unclear until now.

Differential serum and urine metabolites between T2DM patients with or without peripheral neuropathy

In the present study, GC-TOFMS based metabolomics was used to identify related metabolic changes in T2DM patients with and without peripheral neuropathy. We screened out 53 serum metabolites and 56 urine metabolites

Table 6. Serum and urine metabolic pathways that significantly changed among DPN mild, moderate, and severe groups

Sample	Differential Metabolic Pathway	Pathway name	*P*	Impact	Up	Down
Serum	Mod_Mild	Arginine biosynthesis	7.76E-05	0.25	L-Glutamic acid; Ornithine; L-Glutamine	Fumaric acid; Urea
		D-Glutamine and D-Glutamate metabolism	1.18E-02	0.67	L-Glutamic acid; L-Glutamine	
		Alanine, aspartate and glutamate metabolism	1.60E-02	0.32	L-Glutamic acid; L-Glutamine	Fumaric acid; L-Alanine
		Nitrogen metabolism	1.73E-02	0.25	L-Glutamic acid; L-Glutamine	
		Histidine metabolism	1.76e-02	0.33	L-Glutamic acid	1-Methylhistidine; L-Histidine
	Sev_Mild	Glyoxylate and dicarboxylate metabolism	1.06E-02	0.2		Glycolic acid; Glyceric acid; L-Glutamic acid; L-Serine; Isocitric acid
		Galactose metabolism	2.95E-02	0.09		D-Glucose; D-Galactose; Myoinositol; Sucrose
		Starch and sucrose metabolism	4.31E-02	0.12	Fructose 6-phosphate	D-Glucose; Sucrose
		Valine, leucine and isoleucine biosynthesis	4.64E-02	0.25		L-Leucine; Ketoleucine
	Sev_Mod	Glyoxylate and dicarboxylate metabolism	9.31E-03	0.16	Pyruvic acid	Glycolic acid; L-Glutamic acid; L-Serine; Isocitric acid
		Alanine, aspartate and glutamate metabolism	3.01E-02	0.19	Pyruvic acid	L-Glutamic acid; L-Asparagine; N-Acetyl-L-aspartic acid
		Cysteine and methionine metabolism	4.65E-02	0.15	L-Cystine; Pyruvic acid	L-Serine; Homocysteine
Urine	Mod_Mild	Phenylalanine, tyrosine and tryptophan biosynthesis	1.08E-02	0.75	L-Phenylalanine	Phenylpyruvic acid
		Glyoxylate and dicarboxylate metabolism	4.41E-02	0.24	cis-Aconitic acid; Citric acid; Glycolic acid; Glycine	
		Valine, leucine and isoleucine biosynthesis	4.51E-02	0	L-Valine	Ketoleucine
	Sev_Mild	Glyoxylate and dicarboxylate metabolism	1.52E-03	0.32	cis-Aconitic acid; Citric acid; Glycolic acid; Glycine	Glyceric acid; L-Glutamic acid
		Pentose and glucuronate interconversions	3.83E-02	0.14	D-Xylose; L-Arabinose; L-Arabitol	
		Ascorbate and aldarate metabolism	4.26E-02	0.2		Myoinositol; D-Glucaric acid
	Sev_Mod	β-Alanine metabolism	1.58E-02	0.29	Uracil	β-Alanine; L-Histidine; L-Aspartic acid
		Arginine biosynthesis	2.65E-02	0.12	Fumaric acid; Oxoglutaric acid	L-Aspartic acid

Note: Differential metabolic pathways were selected according to $P<0.05$. DPN, Diabetic peripheral neuropathy.

that had significant changes. We focused on the 4 serum metabolites and 6 urine metabolites that changed most significantly. Simultaneously, we also performed MSEA on metabolites in serum and urine, and screened out 6 serum and 9 urinary metabolic pathways in patients with DPN, respectively. It was shown that compared to T2DM patients, serum level of β-alanine and ratio of β-alanine/L-aspartic acid in DPN patients was significantly increased, and compared to mild DPN patients, severe DPN patients had significantly higher serum β-alanine levels and ratio of β-alanine/L-aspartic acid. When present in high levels, β-alanine is a neurotoxin, and damages the brain or nerve tissue. In addition to neurotoxicity, taurine levels in cells can be reduced by β-alanine, which is also necessary for normal respiratory chain function [29]. It is well-known that depletion of cellular taurine reduces respiratory function and increases mitochondrial superoxide production, thereby damaging mitochondria and increasing oxidative stress [30, 31]. Increased oxidative stress leads to a decrease in enzyme activity, which weakens the phosphoryl transfer network and decreases the levels of creatine and pyruvate. Both creatine and pyruvate are important antioxidants and neuroprotective

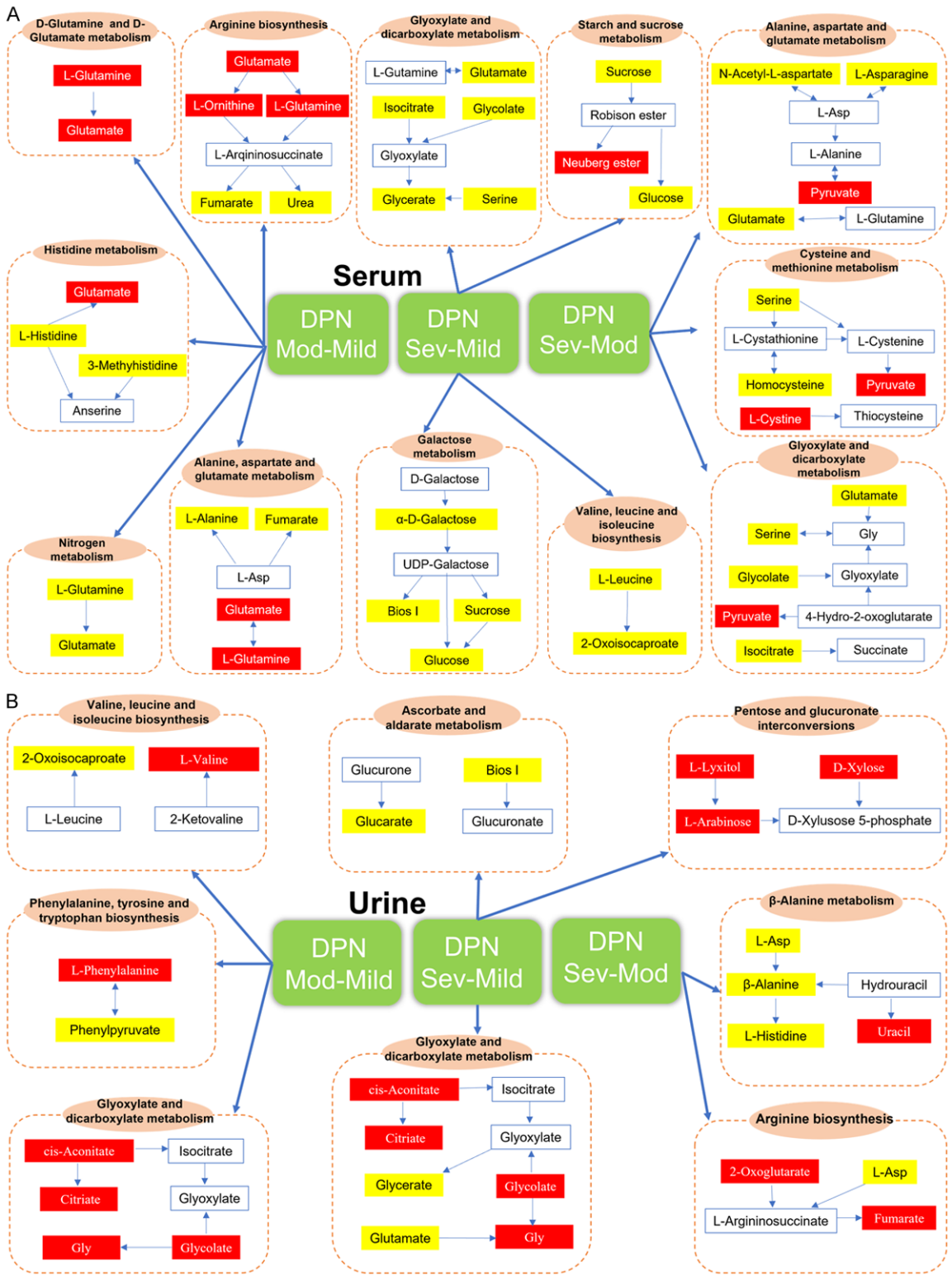

Figure 6. Representative differential metabolic pathways in different degrees of DPN. A. Representative differential metabolic pathways of serum between mild group and moderate group, between mild group and severe group, between moderate group and severe group. B. Representative differential metabolic pathways of urine between mild group and moderate group, between mild group and severe group, between moderate group and severe group. The up-regulated or down-regulated metabolites are highlighted with red and yellow respectively. DPN, Diabetic peripheral neuropathy; L-Asp, L-aspartic acid; L-Arg, L-arginine; Gly, Glyceric acid.

agents [32]. As a result, the weakening of the antioxidant defense capacity will increase the inhibitory effect of the kinase, thereby reducing the content of pyruvate and creatine, and eventually forming a vicious cycle. With the progression of DPN, the continuous accumulation of serum β-alanine and ratio of β-alanine/L-aspartic acid further aggravates the peripheral nerve injury. Therefore, β-alanine and ratio of β-alanine/L-aspartic acid are biomarkers to assess the progress of DPN.

In addition, serum levels of caproic acid in DPN patients were found to be significantly higher than those in T2DM patients without peripheral neuropathy. Caproic acid is a medium-chain fatty acid. Saresella *et al*. [33] confirmed that caproic acid promotes T cell differentiation, thereby aggravating the inflammatory response of the neurological disease multiple sclerosis. This result suggests that the metabolism of caproic acid in the blood of DPN patients may affect the progression of DPN, which is worthy of further exploration. L-arabinose is a five-carbon sugar like D-xylose [34]. L-arabitol is a downstream product of L-arabinose degradation pathway, and L-arabitol inhibited the growth on L-arabinose [35, 36]. Onkenhout *et al*. [37] have pointed out that L-arabinose metabolism is disturbed at the level of L-arabitol degradation due to the lack of L-arabitol dehydrogenase, resulting in the accumulation of L-arabinose and L-arabitol in patients. Due to the toxicity of L-arabitol to the central nervous system, together with our results, the ratio of L-arabinose/L-arabitol was significantly higher in DPN patients compared with T2DM patients without peripheral neuropathy. The ratio of L-arabinose/L-arabitol may be an important factor affecting the progression of DPN. In addition, there are currently no relevant reports in the literature on the relationship between the 6 urine metabolites and DPN.

ROC curve analysis showed that using these four serum metabolites and six urine metabolites could distinguish patients with DPN from T2DM without it, with good accuracy in both serum and urine samples. Collectively, these results further extend our understanding of key regulatory metabolic pathways involved in the pathophysiology of DPN, as well as provide some promising biomarkers for effective DPN early diagnosis.

Differential serum and urine metabolites in T2DM patients with peripheral neuropathy of different degrees

Serum and urine metabolites of three groups of patients with different degrees of DPN were further analyzed. 5 candidate serum biomarkers and 6 candidate urine biomarkers were found to have the most significant changes. Among them, serum hydroxylamine attracted our attention, and it also shows significant differences in different degrees of DPN. Compared with mild and moderate DPN patients, serum hydroxylamine of severe DPN patients was increased significantly. Hydroxylamine is a derivative of ammonium formed during nitrification and anaerobic ammonium oxidation processes [38]. It is a known genotoxic impurity compound [39]. Toxic reactions occur when the body's hydroxylamine concentration is significantly higher than normal cellular metabolism [40]. Therefore, elevated hydroxylamine concentrations may be a factor promoting the progression of DPN.

Serum putrescine and L-kynurenine have significant differences in different degrees of DPN. Compared to patients with mild DPN, patients with moderate DPN have significantly higher levels of putrescine in serum. Elevated putrescine is known to cause neurological impairment [41]. Stewart *et al*. [42] showed that patients with mild cognitive impairment who subsequently developed Alzheimer's disease had 10% higher putrescine. In combination with the above research, we present that putrescine plays important roles in the development of DPN. Tryptophan is a major precursor in kynurenine pathway and it has been discussed in various *in vitro* studies that the metabolites quinolinic acid causes neurotoxicity and kynurenic acid acts as neuroprotectants respectively [43]. Studies have shown that L-kynurenine plays a crucial role in the modulation of pain processing both peripherally and centrally [44]. Our results showed that serum L-kynurenine was significantly elevated in patients with severe DPN compared to patients with mild DPN. We speculate that whether L-kynurenine is protective or toxic to the nervous system is concentration-related, and the role of L-kynurenine in the occurrence and development of DPN remains to be further studied.

Compared to mild DPN patients, urine D-xylose of severe DPN patients increased significantly. Kim *et al*. [45] found that D-xylose regulated blood glucose levels through suppressing phosphoenolpyruvate carboxylase in diabetic rats and enhancing glucose uptake *in vitro*. This further proves that D-xylose may be a factor affecting the progression of DPN. However, other urine metabolites related to the different development degrees of DPN found in this study have not been mentioned in the relevant literature.

Differential metabolic pathways in T2DM patients with or without peripheral neuropathy and DPN patients of different degrees

We found a number of serum and urine metabolic pathways closely related to DPN. By integrating the information of these metabolic pathways, we found that arginine biosynthesis, galactose metabolism, glyoxylate and dicarboxylate metabolism were the main pathways involved in serum metabolism as well as important pathways in urinary metabolism in T2DM patients with or without peripheral neuropathy. We also found that glyoxylate and dicarboxylate metabolism and valine, leucine, and isoleucine biosynthesis were the main pathways involved both in serum metabolism and urine metabolism in DPN of different degrees. Glyoxylate and dicarboxylate metabolism may be involved not only in the occurrence but also in the progression of DPN. In future research, we will focus on the above related metabolic pathways, and explore key factors regulating progression of DPN.

Limitations

There are some limitations in our current research. First, because different instruments for detecting metabolites have their own limitations, it is impossible to detect all metabolites in serum and urine. Second, there were many metabolites and metabolic pathways screened in this research. Although the data are comprehensive, the research depth is not enough. Third, in order to understand the precise biological processes that lead to the development of DPN by metabolites, it is necessary to further study the relationship between metabolites and related metabolic pathways in combination with other omics techniques or mechanism study.

Conclusion

This study used GC-TOFMS technology to conduct non-targeted metabolomic studies on serum and urine samples of patients with DPN and T2DM. Our key finding is that the occurrence and development of disease in DPN patients are associated with metabolism disorders of amino acids, carbohydrates, fatty acids, and organic acids. In summary, compared to T2DM patients, DPN patients have higher serum levels of β-alanine, ratio of β-alanine/L-aspartic acid, caproic acid, and ratio of L-arabinose/L-arabitol. Compared to mild DPN patients, severe DPN patients have lower serum levels of β-alanine, ratio of β-alanine/L-aspartic acid, L-kynurenine, and hydroxylamine. In addition, we found that glyoxylate and dicarboxylate metabolism was a differential metabolic pathway not only between T2DM and DPN, but also in different degrees of DPN. Further understanding of the role of β-alanine, D-xylose, and other differential metabolites as well as the role of glyoxylate and dicarboxylate metabolic pathway in the pathogenesis of DPN patients may be of great significance for the preliminary clinical screening and diagnosis of DPN.

Acknowledgements

The metabolites involved in this paper can be found in HMDB (http://www.hmdb.ca/), and the metabolic pathway can be found in KEGG (http://www.kegg.jp/). This work was supported by the National Natural Science Foundation of China (81620108030, 81873076) and the Hundred Talents Program from Shanghai University of Traditional Chinese Medicine.

Disclosure of conflict of interest

None.

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Table S1. Toronto clinical scoring system (TCSS)

A. Neurological Symptom Questionnaire Score				
	Date _____		Date _____	
Do you feel numbness in your legs or feet?	No□ (0)	Yes□ (1)	No□ (0)	Yes□ (1)
Do you have pain, tenderness or burning in your legs or feet?	No□ (0)	Yes□ (1)	No□ (0)	Yes□ (1)
Do you have a prickly sensation in your legs or feet?	No□ (0)	Yes□ (1)	No□ (0)	Yes□ (1)
Do you feel weak in your legs or feet?	No□ (0)	Yes□ (1)	No□ (0)	Yes□ (1)
Do you feel that your gait is unstable when walking?	No□ (0)	Yes□ (1)	No□ (0)	Yes□ (1)
Do the upper limbs have similar symptoms as above?	No□ (0)	Yes□ (1)	No□ (0)	Yes□ (1)
Total score				
B. Neural reflex score				
	Date _____		Date _____	
	Left side	Right side	Left side	Right side
Ankle reflex Yes (0) Weaken (1) No (2)				
Knee reflex Yes (0) Weaken (1) No (2)				
Total score				
C. Sensory function test score				
	Date _____		Date _____	
Needle sensation on the back of the great toe R □ L □	Pain□ (0)	No pain□ (1)	Pain□ (0)	No pain□ (1)
Vibration sensation in the great toe R □ L □	Pain□ (0)	No pain□ (1)	Pain□ (0)	No pain□ (1)
Light touch on the back of the great toe R □ L □	Pain□ (0)	No pain□ (1)	Pain□ (0)	No pain□ (1)
Temperature sensation on the back of the great toe R □ L □	Pain□ (0)	No pain□ (1)	Pain□ (0)	No pain□ (1)
Position sense of the great toe R □ L □	Pain□ (0)	No pain□ (1)	Pain□ (0)	No pain□ (1)
Total score				
Cumulative total score (A+B+C):				
Grade:				

(0 to 5 points: no DPN, 6 to 8 points are mild DPN, 9 to 11 points are moderate DPN, and 12 to 19 points are severe DPN).
Note: TCSS was proposed by Perkins *et al*. in 2001 (*Simple screening tests for peripheral neuropathy in the diabetes clinic. Diabetes care 2001, 24 (2), 250-6*). It uses neuroelectro-physiological examination as the gold standard for the diagnosis of DPN, which has high diagnostic value for DPN and clinical application value in the classification of neuropathy. TCSS score includes three parts: neurologic symptom score, neurologic reflex score, and sensory function test score. Neurological symptoms include numbness, pain, weakness, needle-like sensation in the lower extremities, walking instability, and similar symptoms in the upper extremities. Nerve reflex includes ankle reflex and knee reflex, scored on both sides, 0 points for normal, 1 point for attenuation, 2 points for disappearance, a total of 8 points; sensory function examination included pain sensation, pressure sensation, temperature sensation, vibration sensation, and position sensation of the right big toe, with a score of 0 for normal and 1 for abnormal, with a total score of 5 and a total score of 19. According to the grading criteria of TCSS, DPN is absent with a score of 0 to 5, mild with a score of 6 to 8, moderate with a score of 9 to 11, and severe with a score of 12 to 19. DPN, diabetic peripheral neuropathy; TCSS, Toronto clinical scoring system.

Table S2. Clinical information of T2DM patients with and without peripheral neuropathy

Characteristic	T2DM (n=41)	DPN (n=74)			
		Overall	DPN-Mild (n=37)	DPN-Moderate (n=24)	DPN-Severe (n=13)
Gender (M/F)	29/12	46/28	19/18	19/5	8/5
Age (years)	71 (55-79)	64 (58.75-74)	63 (55.5-71)	66 (60-76)	62 (60-79)
BMI (kg/m^2)	24.34 (22.86-26.12)	24.49 (23.2-27.04)	24.04 (22.49-26.42)	24.57 (23.51-26.69)	26.2 (24.49-28.13)
WHR	0.93 (0.91-0.96)	0.95 (0.93-0.98)	0.95 (0.9-0.97)	0.94 (0.94-0.99)	0.95 (0.95-0.99)
Glucose (mmol/L)	7.5 (5-9.6)	7.6 (5.57-9.55)	7.42 (5.24-9.03)	7.2 (6.02-8.86)	8.97 (6.46-10.6)
2hPG (mmol/L)	11 (6.98-14.53)	11.54 (8.27-14.27)	13.46 (9.19-14.8)	9.86 (8.02-13)	8.41 (6.89-12.1)
C-peptide (nmol/L)	1.41 (0.88-2.01)	0.71 (0.43-1.01)***	0.9 (0.48-1.12)	0.63 (0.43-0.8)	0.46 (0.35-0.67)
2hC-peptide (nmol/L)	1.96 (1.07-4.01)	1.34 (0.8-2.75)	1.55 (0.94-3.12)	1.38 (0.94-2.45)	0.8 (0.51-1.5)
Insulin (mmol/L)	69.74 (44.59-107.73)	12.36 (5.56-40.12)***	11.15 (5.56-37.2)	11.8 (6.8-26.41)	37.52 (23.1-52.93)
HbA1C (mg/dl, %)	8.4 (6.98-9.58)	8.5 (7.05-10)	8.5 (7.2-10.25)	8.1 (7-8.9)	9.25 (7.53-10.25)
WBC (*10^9/L)	7.36 (6.24-8.62)	6.35 (5.7-7.65)	6.45 (5.9-8.7)	6.2 (5.55-7.45)	6.6 (5.4-7.2)
Neu (%)	63.1 (58.6-71.5)	66.2 (60.98-72.28)	65.45 (60.78-71.3)	69.2 (65.75-73.15)$^{\#\#}$	61.5 (59.2-63.6)
Lym (%)	23.5 (17.5-29.3)	22.85 (17.45-29.6)	23.85 (17.45-30)	20.6 (16.5-24.3)	28.7 (21.3-29.8)
Mon (%)	8 (7.1-9.8)	6.95 (5.68-8.1)**	6.6 (5.5-8.13)	6.9 (5.75-7.8)	7.9 (6.3-8.2)
Eso (%)	2.2 (1.6-3.5)	1.55 (0.8-2.73)*	1.55 (0.75-2.83)	1.3 (0.5-2.05)	2.2 (1.1-2.9)
Bas (%)	0.3 (0.1-0.4)	0.4 (0.3-0.6)**	0.35 (0.3-0.5)	0.4 (0.3-0.55)	0.6 (0.5-0.7)
RBC (*10^12/L)	4.03 (3.73-4.39)	4.29 (3.96-4.78)*	4.23 (3.73-4.6)	4.3 (4.1-4.51)	4.55 (3.92-4.91)
HGB (g/L)	117 (102-126)	131 (118-143.25)***	125.05 (105.75-144)	131 (125-140)	133 (119-144)
HCT (%)	34.6 (31.2-36.3)	0.45 (0.4-33)***	10.3 (0.4-33.48)	0.45 (0.4-36.1)	0.43 (0.37-0.45)
MCV (%)	86.6 (82.9-89.7)	89.8 (86.93-92.45)***	89.1 (86.45-92.1)	90.2 (88.7-91.9)	90.8 (86.5-94)
HGB-Mass (pg)	29.3 (27.8-30.4)	30.1 (29.2-31.25)**	29.9 (28.8-31.1)	30.4 (29.85-31.3)	30.1 (29.7-31.4)
HGB-Conc (g/L)	335 (329-345)	331 (327.75-341)	331.5 (324.5-341.25)	333 (330-342)	330 (329-332)
RDW-CV (%)	13.5 (12.7-14.5)	13 (12.6-13.93)	12.85 (12.4-14.15)	13 (12.7-13.45)	13.1 (12.6-13.9)
PLT (*10^9/L)	265 (196-304)	187 (151.75-215.25)***	191 (171.5-218)	181 (144-203)	201 (164-229)
MPV (fL)	10.4 (9.7-11.1)	9.9 (8.5-11.03)	10.35 (9.13-11.03)	9.4 (8.2-11.45)	9.1 (8.5-9.9)
hs-CRP (mg/L)	8 (4-39)	0.72 (0.5-1.48)***	0.82 (0.5-2.42)	0.57 (0.5-1)	0.75 (0.6-1)
TP (g/L)	65.8 (62.3-68.9)	65.79 (61.4-69.68)	68.1 (60-70)	64.62 (62-66.92)	65.24 (62.4-69.4)
ALB (g/L)	35.8 (32.3-37.2)	38.79 (35.48-42.37)***	38 (35-42.6)	38.99 (35.38-42.34)	40.66 (37-42)
GLB (g/L)	30.4 (27.6-34.9)	26 (24-28.6)***	27 (24-29)	26 (24-26.45)	26 (23.9-28)
A/G	1.1 (1-1.3)	1.46 (1.3-1.73)***	1.42 (1.19-1.68)	1.48 (1.4-1.75)	1.55 (1.47-1.73)
AST (U/L)	14 (11-22)	19.5 (15-27.75)**	20 (16.2-27)	18.1 (15-25.43)	20 (12-32)
ALT (U/L)	17 (13-21)	20.5 (16-25)**	20 (16-28)	21.5 (16.75-24.5)	20 (16-22)
AST_ALT	1.11 (0.83-1.4)	1 (0.81-1.38)	1 (0.88-1.43)	1.07 (0.86-1.23)	1 (0.69-1.25)
r-GT (U/L)	23 (16-34)	23.1 (15.46-33.61)	24 (16-32)	22.61 (14.37-50.58)	23.2 (17.56-28.03)
AKP (U/L)	98 (78-119)	77 (63-95)***	86 (62-102)	69 (60.5-84)	75 (72-94)
TBIL (μmol/L)	7.9 (5.8-8.6)	11.35 (8.08-17.2)***	11.3 (7.1-16)	12.99 (9.18-20.52)	9.8 (8.1-15)
DBIL (μmol/L)	3 (2.1-3.7)	2 (0.9-3.12)***	1.51 (0.8-2.9)	2.4 (1.56-3.99)	2 (1.58-3)
TBA (μmol/L)	3.3 (2.7-4.3)	4 (3-5.77)	4.12 (2.96-6.25)	4.74 (3.01-5.81)	3 (2.17-3.91)
Cr (μmol/L)	71 (54-93)	68 (57-82)	63 (56.3-82)	73.06 (58.32-87.01)	68 (59.5-72)
Urea (mmol/L)	6.8 (4.6-8)	6.05 (5.06-7.31)	6.58 (5.1-8.33)	5.71 (4.63-7.06)	5.89 (5.2-7.16)
UA (μmol/L)	256 (207-326)	321 (253-376)*	329 (253.4-419)	321 (229.3-369.5)	278 (258-362.2)
Ca (mmol/L)	2.24 (2.16-2.4)	2.23 (2.17-2.32)	2.25 (2.12-2.33)	2.22 (2.19-2.26)	2.24 (2.17-2.39)
P (mmol/L)	1.22 (1.1-1.36)	1.22 (1.05-1.33)	1.26 (1.11-1.43)	1.13 (1.01-1.26)	1.23 (1.21-1.3)
K (mmol/L)	4 (3.8-4.2)	4.1 (3.82-4.4)	4.1 (3.82-4.33)	3.98 (3.73-4.35)	4.21 (3.91-4.7)
Na (mmol/L)	139 (137-140)	139.71 (137.45-141)	139.5 (137.83-141)	140 (137.38-142.07)	139 (137.2-140.88)
Cl (mmol/L)	102 (100-104)	103 (100.25-105.38)	103 (100-104.12)	103.9 (100.62-106.91)	102 (101-104.62)
TG (mmol/L)	1.05 (0.85-1.37)	3.86 (1.52-4.86)***	3.81 (1.49-5.06)	3.57 (1.49-4.57)	4.04 (3.3-4.75)
TC (mmol/L)	3.92 (3.53-5.09)	2.6 (1.55-4.21)***	3.48 (2.01-4.51)	2.75 (1.4-4.03)$^{\Delta\Delta}$	1.36 (0.99-1.84)
HDL (mmol/L)	1.01 (0.84-1.17)	1 (0.85-1.13)	0.91 (0.83-1.06)	1.04 (0.9-1.21)	1.08 (0.88-1.16)
LDL (mmol/L)	2.52 (2.06-3.29)	2.56 (2.05-3.12)	2.65 (2.23-3.21)	2.47 (1.76-3.11)	2.64 (2.06-2.92)
ApoA (g/L)	1.06 (0.9-1.25)	1.11 (0.96-1.22)	1.09 (0.93-1.19)	1.12 (1.07-1.32)	1.11 (1-1.17)

ApoB (g/L)	0.77 (0.62-0.88)	0.88 (0.74-1.01)*	0.93 (0.8-1.02)	0.81 (0.68-1.18)	0.83 (0.68-0.89)
Lp-A (mmol/l)	225 (108-403)	97.04 (58.64-210.5)*	119.73 (59.36-216.44)	74.26 (59-209)	98.16 (49.76-198.9)

Note: Data are expressed as the medians (IQRs) at normal distribution. The Wilcoxon test was used to compare these variables between T2DM patients with and without peripheral neuropathy, and $P<0.05$ was considered significant. The ANOVA test was used to compare these variables among the three degrees of DPN. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, Comparison of T2DM patients and DPN patients; ##, $P<0.01$, Comparison of severe DPN patients with moderate DPN patients; ΔΔ, $P<0.01$, Comparison of severe DPN patients with mild DPN patients. M, male; F, female; BMI, body mass index; WHR, waist-to-hip Ratio; 2hPG, two hours post-load plasma glucose; HbA1C, glycosylated hemoglobin or glycated hemoglobin; WBC, white blood cell; Neu, neutrophil ratio; Lym, lymphocyte ratio; Mon, monocyte ratio; Eso, eosinophil ratio; Bas, basophil ratio; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; HGB-Mass, RBC average HGB content; HGB-Conc, RBC average HGB concentration; RDW-CV, red blood cell distribution width coefficient of variation; PLT, platelet; MPV, mean platelet volume; hs-CRP, hypersensitive C-reactive protein; TP, total protein; ALB, albumin; GLB, globulin; A-G, albumin/globulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AST-ALT, aspartate aminotransferase/alanine aminotransferase; r-GT, Glutamyl transpeptidase; AKP, alkaline phosphatase; TBIL, total bilirubin; DBIL, direct bilirubin; TBA, total bile acid; Cr, creatinine; UA, uric acid; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ApoA, apolipoprotein A; ApoB, apolipoprotein b; Lp-A, lipoprotein A; T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy; ANOVA, Wilcoxon test and analysis of variance.

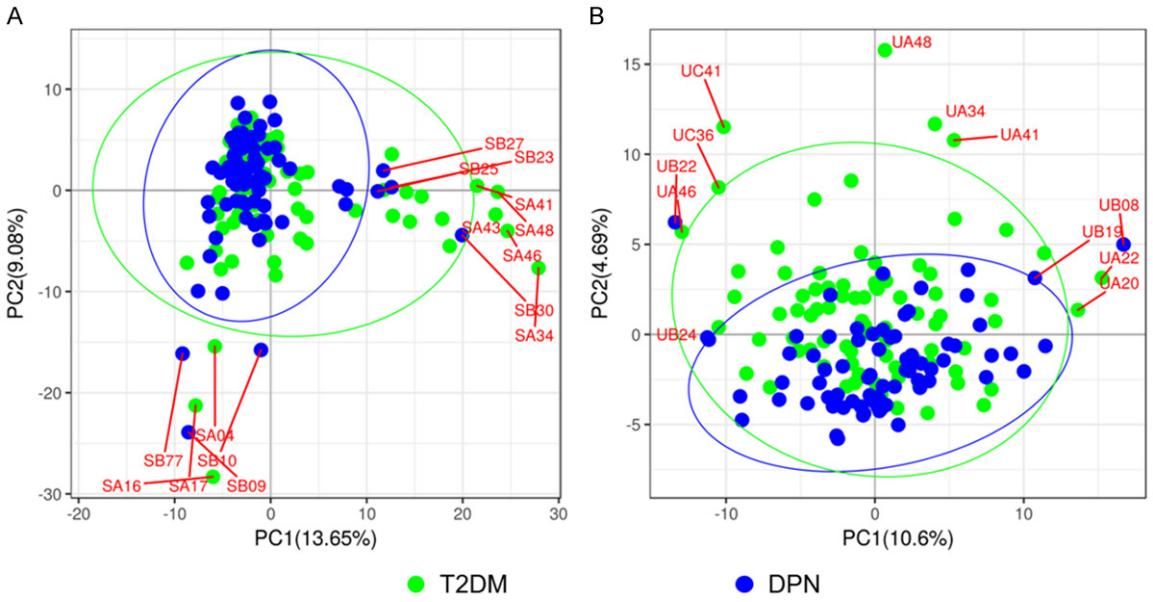

Figure S1. PCA score plot of T2DM patients with or without peripheral neuropathy. A. PCA score plot of serum. B. PCA score plot of urine. T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy; PCA, principal component analysis.

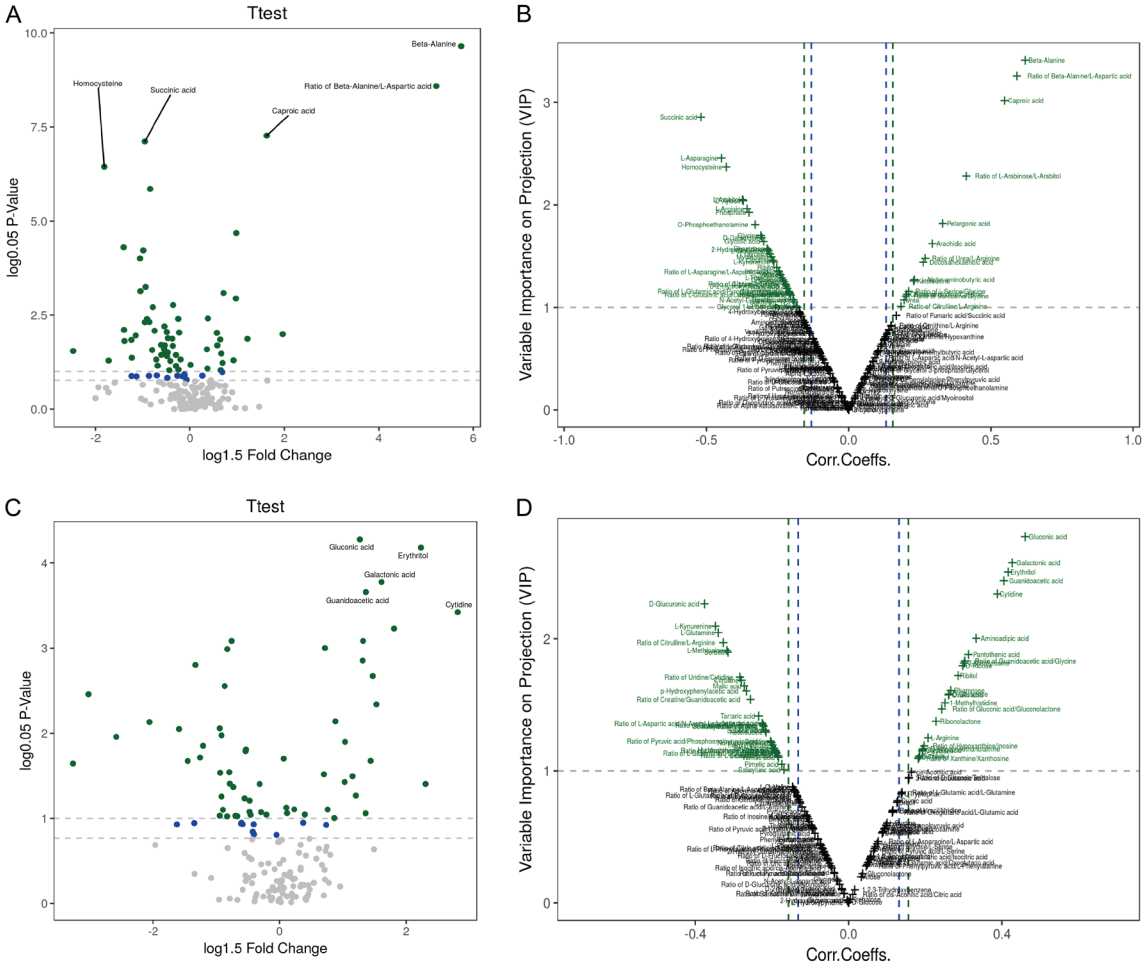

Figure S2. Visualization of differential metabolite profiles. A. Visualization of differential metabolite profiles using T-test in serum. B. Visualization of differential metabolite profiles using V-plot in serum. C. Visualization of differential metabolite profiles using T-test in urine. D. Visualization of differential metabolite profiles using V-plot in urine.

Table S3. Differential serum metabolites between T2DM patients with and without peripheral neuropathy

Class	Name	T2DM	DPN	*P*	VIP	FC	HMDBID
Amino Acid	β-Alanine	8465 [6411.5, 12577]	86890.5 [10234.75, 124466]	2.78E-13	3.4	10.3	HMDB00056
	Ratio of β-Alanine/L-Aspartic acid	0.07 [0.044, 0.103]	0.57 [0.062, 1.036]	6.69E-12	3.3	8.3	HMDB00056/HMDB00191
	Ratio of Urea/L-Arginine	0.04 [0.028, 0.066]	0.09 [0.055, 0.204]	2.55E-03	1.5	2.2	HMDB00294/HMDB00517
	Ketoleucine	101062.5 [75295.5, 126695.25]	127374.5 [105941.25, 160081.5]	2.30E-03	1.3	1.3	HMDB00695
	L-α-aminobutyric acid	45440 [34161, 66585.25]	58918 [46082, 75829.5]	3.91E-03	1.3	1.3	HMDB00452
	Ratio of Ketoleucine/L-Leucine	0.19 [0.145, 0.27]	0.24 [0.192, 0.322]	6.23E-03	1.2	1.3	HMDB00695/HMDB00687
	Ratio of L-Serine/Glycine	0.21 [0.153, 0.265]	0.24 [0.2, 0.293]	8.55E-03	1.2	1.2	HMDB00187/HMDB00123
	L-Glutamine	2598867.5 [2238690, 2859982.75]	2249147 [1989705.5, 2595700.75]	2.51E-04	1.6	0.9	HMDB00641
	L-Threonine	252817.5 [202675.5, 369540.75]	224418.5 [171236.5, 281975.75]	1.34E-02	1.3	0.9	HMDB00167
	Ratio of Glycine/L-Serine	4.8 [3.774, 6.536]	4.15 [3.414, 5.011]	6.48E-03	1.2	0.9	HMDB00123/HMDB00187
	Ornithine	1912452 [1569777.75, 2290531.5]	1765360 [1408023.75, 2016804]	1.91E-02	1.2	0.9	HMDB00214
	Dimethylglycine	360527 [277457.5, 479780]	320145.5 [226290.75, 410235.25]	3.21E-02	1.2	0.9	HMDB00092
	Glycine	1576748.5 [1388353.5, 1938836.25]	1305012 [1037587, 1764428]	3.52E-03	1.7	0.8	HMDB00123
	Ratio of L-Glutamic acid/Pyroglutamic acid	0.2 [0.133, 0.294]	0.16 [0.114, 0.227]	5.98E-03	1.2	0.8	HMDB00148/HMDB00267
	D-2-Hydroxyglutaric acid	7223.5 [5250.5, 9489.25]	5814 [4554.5, 7972]	6.17E-03	1.2	0.8	HMDB00606
	Ratio of L-Glutamic acid/Oxoglutaric acid	39.57 [20.864, 82.624]	31.7 [20.147, 40.908]	8.44E-03	1.1	0.8	HMDB00148/HMDB00208
	4-Hydroxyproline	84771 [52330.75, 135263]	69592 [41561.25, 108253.75]	1.47E-02	1.1	0.8	HMDB00725
	L-Asparagine	129961 [98746, 160082.5]	92289 [72519, 128003.75]	2.42E-08	2.5	0.7	HMDB00168
	Citrulline	118190 [78924.5, 139380.5]	83332.5 [66192.25, 103501.25]	9.73E-04	1.5	0.7	HMDB00904
	L-Kynurenine	1774 [1260.75, 2512.25]	1309.5 [1089.75, 1627.5]	1.95E-03	1.4	0.7	HMDB00684
	Ratio of L-Asparagine/L-Aspartic acid	0.93 [0.683, 1.42]	0.64 [0.512, 0.859]	9.87E-04	1.3	0.7	HMDB00168/HMDB00191
	1-Methylhistidine	9527 [6758, 17302.75]	6892 [4879, 9575]	9.02E-03	1.2	0.7	HMDB00001
	L-Arginine	121134.5 [75156.25, 170189.5]	68611.5 [55602.75, 108968.25]	2.53E-06	2	0.6	HMDB00517
	N-Acetyl-L-aspartic acid	28959 [12100.75, 85308.75]	16515 [11504.75, 33267.75]	4.40E-03	1.1	0.6	HMDB00812
	Homocysteine	2231 [1438.5, 3624]	1070.5 [814.25, 1519.75]	4.12E-09	2.4	0.5	HMDB00742
Carbohydrates	Ratio of L-Arabinose/L-Arabitol	0.36 [0.183, 0.483]	0.53 [0.411, 0.723]	8.12E-07	2.3	1.5	HMDB00646/HMDB01851
	Allose	209974 [161515.75, 272778]	180493 [138060, 230997]	3.67E-03	1.3	0.9	HMDB01151
	D-Galactose	1343977 [773314.5, 2472425.75]	1101330.5 [494351, 1758685]	2.16E-03	1.7	0.8	HMDB00143
	D-Mannose	1237605 [779105, 1558149.5]	1001003 [560088.25, 1329826]	1.05E-02	1.2	0.8	HMDB00169
	Erythritol	46885 [29772, 86767]	38676 [27456.25, 54065.75]	1.09E-02	1.1	0.8	HMDB02994
	D-Threitol	7897.5 [4058.25, 14192.25]	6374.5 [4397.25, 9862.5]	1.10E-02	1.1	0.8	HMDB04136
	Threonic acid	49101 [34642, 82586.75]	38426 [27637.75, 49902.25]	1.33E-02	1.1	0.8	HMDB00943
	L-Arabitol	70136.5 [54332.25, 132439.25]	45737 [38245.75, 57762.75]	6.11E-06	2.1	0.7	HMDB01851
	D-Xylose	14459 [11304, 19584.5]	9691 [7973.25, 12106.25]	3.26E-06	2	0.7	HMDB00098
	Rhamnose	35064 [28557.25, 45258.25]	25501 [18714.75, 35308.75]	2.96E-04	1.6	0.7	HMDB00849
	Isomaltose	5300.5 [2986.5, 11944.75]	3012.5 [1827.75, 4863.75]	1.82E-03	1.4	0.6	HMDB02923
	D-Maltose	19762.5 [10610, 37502.5]	11950 [8645.75, 17745]	4.04E-03	1.3	0.6	HMDB00163
	Ribitol	8493.5 [5480, 13316]	3117.5 [1806, 5450.25]	9.74E-03	1.4	0.4	HMDB00508

Fatty Acids	Caproic acid	2086 [1458.75, 2718.5]	4034 [2869.5, 5441.75]	3.46E-10	3	1.9	HMDB00535
	Arachidic acid	2287.5 [1383, 3384.25]	3393 [2080.25, 4991.75]	1.49E-04	1.6	1.5	HMDB02212
	Pelargonic acid	4981 [3767.5, 6606.75]	6649 [4791, 10595.5]	9.61E-05	1.8	1.3	HMDB00847
	Docosahexaenoic acid	7163 [3949.75, 10173.5]	8358.5 [6433, 13492.75]	7.34E-04	1.4	1.2	HMDB02183
Organic Acids	L-Pipecolic acid	2488.5 [1777, 3329]	1887.5 [1538, 2368]	3.15E-02	1.1	0.8	HMDB00716
	Succinic acid	14853.5 [11407, 18298.25]	10089.5 [8349, 12744]	5.54E-10	2.9	0.7	HMDB00254
	Glycolic acid	77425.5 [56738, 99468.5]	53557.5 [42387.75, 70281]	7.71E-04	1.6	0.7	HMDB00115
Nucleotide	Ratio of Uridine/Cytidine	4.37 [2.142, 7.544]	7.16 [4.136, 11.665]	3.70E-03	1.1	1.6	HMDB00296/HMDB00089
	Cytidine	556.5 [381.75, 955.5]	349.5 [277, 440.5]	2.84E-03	1.3	0.6	HMDB00089
Alcohols	2-Hydroxypyridine	340808.5 [296556.75, 378074]	308389 [276739.75, 351678.75]	7.59E-04	1.6	0.9	HMDB13751
	Myoinositol	248754.5 [140870, 755458.5]	226877.5 [163312.5, 329881.75]	2.25E-03	1.5	0.9	HMDB00211
	Glycerol	461421.5 [342061.25, 730280]	370640.5 [266600.25, 500060.25]	1.38E-03	1.5	0.8	HMDB00131
Lipids	Cholesterol	889214.5 [517172, 1121250.25]	894017 [570549, 1704304]	2.94E-02	1.1	1	HMDB00067
	O-Phosphoethanolamine	20268.5 [13620.25, 26417.75]	13852.5 [9357.25, 18844.75]	5.94E-05	1.8	0.7	HMDB00224
Phosphate	Phosphate	1426649.5 [885174.5, 1733174]	933315 [577265.5, 1207500]	8.27E-05	1.9	0.7	HMDB01429

Note: Differential metabolites were selected according to VIP>1 and *P*<0.05. Values are expressed as medians (IQR). *P* values were calculated from t test for continuous variables and adjusted by FDR method; VIP, variable influence on projection; FC, fold change; HMDB, The Human Metabolome Database; T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy.

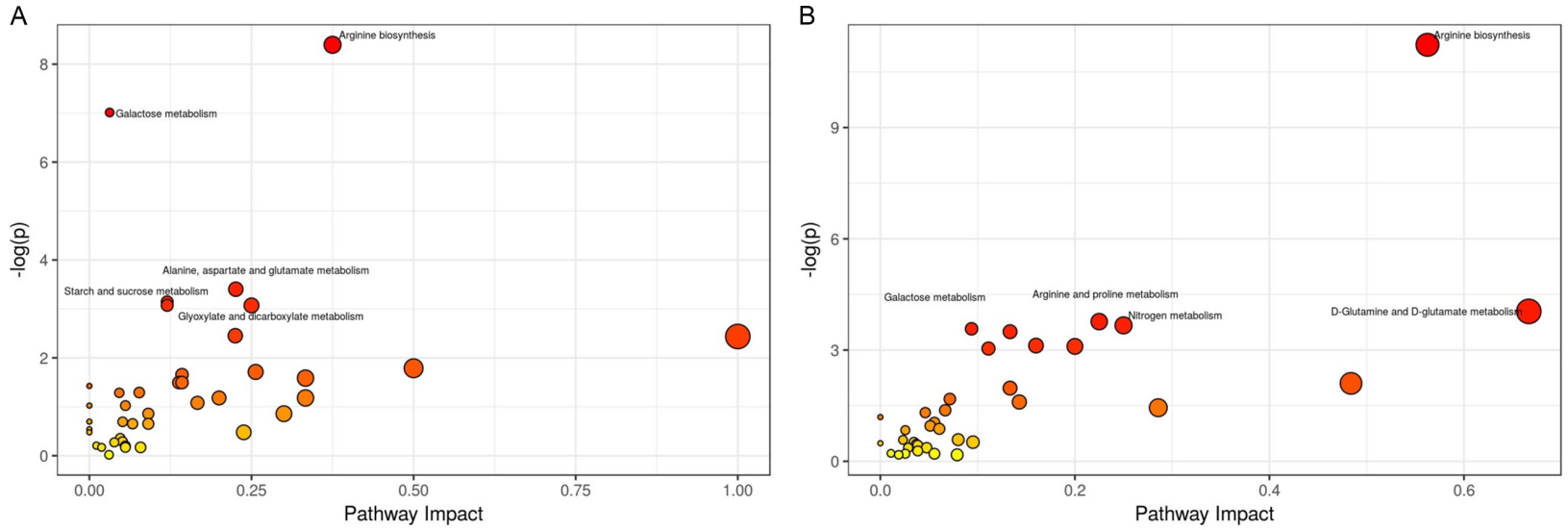

Figure S3. Overview of metabolic pathway enrichment analysis. A. Pathway analysis of identified serum metabolites between T2DM patients with and without peripheral neuropathy. The x-axis represents the pathway impact and the y-axis represents the pathway enrichment. Larger sizes and darker colors represent higher pathway enrichment and higher pathway impact values. B. Pathway analysis of the identified urinary metabolites between T2DM patients with and without peripheral neuropathy.

Table S4. Differential urinary metabolites between T2DM patients with and without peripheral neuropathy

Class	Name	T2DM	DPN	*P*	VIP	FC	HMDBID
Amino Acid	Guanidoacetic acid	108600 [45783.25, 163194.25]	188864 [107721, 271065]	1.74E-05	2.4	1.7	HMDB00128
	L-Kynurenine	31975 [16180.5, 48045.75]	18652 [12982, 29713]	2.26E-04	2.1	0.6	HMDB00684
	Aminoadipic acid	66668 [27833.75, 96748.25]	138729 [81348, 265463]	6.27E-05	2	2.1	HMDB00510
	L-Glutamine	571266.5 [292141.25, 1132363.25]	389124 [217335, 681003]	2.10E-03	2	0.7	HMDB00641
	Ratio of Citrulline/L-Arginine	6.63 [2.452, 21.47]	2.88 [1.324, 4.617]	1.69E-03	2	0.4	HMDB00904/HMDB00517
	L-Methionine	72596 [47719.25, 94857.75]	51072 [36083, 68495]	4.75E-04	1.9	0.7	HMDB00696
	3-Nitrotyrosine	75656 [36678.75, 142895.25]	140600 [80375, 224265]	9.05E-04	1.8	1.9	HMDB01904
	Ratio of Guanidoacetic acid/Glycine	0.54 [0.252, 1.307]	0.97 [0.58, 1.844]	6.62E-03	1.8	1.8	HMDB00128/HMDB00123
	Citrulline	141685.5 [113123.75, 183645.25]	113990 [81362, 161470]	4.68E-03	1.7	0.8	HMDB00904
	1-Methylhistidine	225900 [140604.5, 347182.5]	385599 [232378, 514693]	9.72E-05	1.5	1.7	HMDB00001
	Ratio of Creatine/Guanidoacetic acid	22.02 [7.4, 43.653]	7.73 [3.767, 15.759]	2.84E-03	1.5	0.4	HMDB00064/HMDB00128
	Ratio of L-Aspartic acid/N-Acetyl-L-aspartic acid	0.44 [0.267, 0.678]	0.3 [0.185, 0.515]	2.68E-03	1.4	0.7	HMDB00191/HMDB00812
	L-Aspartic acid	35881.5 [22066, 59149.75]	26013 [18504, 41789]	9.86E-03	1.4	0.7	HMDB00191
	4-Hydroxy-L-proline	197416 [120875.75, 399489.5]	159257 [79097, 232252]	4.43E-03	1.3	0.8	HMDB06055
	Ketoleucine	28046 [15216.75, 95835.75]	16847 [11787, 33590]	5.88E-03	1.3	0.6	HMDB00695
	β-Alanine	48569 [25801.25, 98696.75]	43151 [24785, 61449]	4.02E-02	1.2	0.9	HMDB00056
	Creatine	1740952 [1085163.25, 2834868.75]	1189122 [889060, 2015672]	1.00E-02	1.2	0.7	HMDB00064
	L-Glutamic acid	90836 [56219.25, 146283.75]	83497 [66107, 108869]	4.39E-02	1.1	0.9	HMDB00148
	Ratio of L-Glutamine/L-Glutamic acid	5.18 [2.481, 12.461]	4.3 [2.293, 9.638]	4.41E-02	1.1	0.8	HMDB00641/HMDB00148
	Ratio of L-Serine/Glycine	1.85 [1.442, 2.525]	1.4 [0.942, 1.893]	4.57E-02	1.1	0.8	HMDB00187/HMDB00123
Carbohydrates	Gluconic acid	1208349 [569494.75, 1671856]	2019801 [1220345, 3906582]	2.72E-06	2.8	1.7	HMDB00625
	Galactonic acid	181093.5 [106851.25, 277908.25]	347865 [168861, 541480]	1.22E-05	2.6	1.9	HMDB00565
	Erythritol	937647 [463078.25, 1818760.5]	2319943 [1230673, 4796622]	3.65E-06	2.5	2.5	HMDB02994
	D-Glucuronic acid	1368536.5 [892379.25, 1562827.25]	1006489 [270169, 1326414]	9.72E-05	2.3	0.7	HMDB00127
	Sorbitol	873906.5 [588939.5, 1636230]	625468 [433579, 823296]	1.30E-04	1.9	0.7	HMDB00247
	D-Ribose	905875 [560606, 1176215.75]	1210629 [857018, 1477660]	1.25E-04	1.8	1.3	HMDB00283
	Ribitol	647818 [300532.75, 1098419.5]	985382 [657522, 1514916]	3.40E-03	1.7	1.5	HMDB00508
	Rhamnose	249626.5 [157436.25, 426348.5]	453336 [295528, 587123]	3.34E-04	1.6	1.8	HMDB00849
	D-Galactose	653704.5 [311276.75, 998890]	991912 [603937, 1439106]	1.39E-02	1.6	1.5	HMDB00143
	Ratio of Gluconic acid/Gluconolactone	3.78 [2.111, 10.524]	9.64 [3.144, 22.259]	1.49E-02	1.5	2.5	HMDB00625/HMDB00150
	Ribonolactone	674959 [357334, 894093.5]	896144 [502309, 1177473]	1.06E-02	1.4	1.3	HMDB01900
	Mannitol	1067486 [507564.75, 1724839.5]	763330 [447841, 1256321]	4.66E-02	1.3	0.7	HMDB00765
	D-Xylose	1645113 [1189431, 2374432.25]	2352596 [1662314, 2756137]	1.64E-03	1.2	1.4	HMDB00098
	Ratio of L-Arabinose/L-Arabitol	1.99 [0.931, 4.939]	2.19 [1.203, 3.324]	3.64E-02	1.2	1.1	HMDB00646/HMDB01851
	Sucrose	2023126.5 [694046.25, 4102968.25]	3510774 [1644964, 5830899]	4.18E-02	1.1	1.7	HMDB00258
	D-Threitol	147600.5 [95779.25, 214220.75]	173413 [96569, 289980]	4.37E-02	1.1	1.2	HMDB04136

Organic Acids	Oxalic acid	41113 [27553.75, 79517.75]	67006 [33727, 106252]	2.25E-02	1.6	1.6	HMDB02329
	p-Hydroxyphenylacetic acid	648819 [336995.5, 1253203.25]	570892 [438813, 975425]	1.49E-02	1.6	0.9	HMDB00020
	Malic acid	138996 [78327.75, 267174.75]	73000 [44226, 129016]	2.15E-03	1.6	0.5	HMDB00744
	Tartaric acid	118017.5 [45693.25, 260015.25]	31421 [20490, 85309]	7.27E-03	1.4	0.3	HMDB00956
	Suberic acid	23553.5 [14080.75, 37263.25]	16234 [12514, 23766]	2.32E-02	1.3	0.7	HMDB00893
	Guanidinosuccinic acid	209922.5 [113643.75, 330511.5]	128523 [80558, 220962]	3.89E-03	1.3	0.6	HMDB03157
	Glycolic acid	1538072.5 [792046.75, 2500266.5]	2092364 [1331955, 2854604]	3.66E-02	1.2	1.4	HMDB00115
	Uric acid	329676.5 [263985, 544064.25]	338516 [246198, 438528]	6.12E-03	1.2	1	HMDB00289
	Hydroxyphenyllactic acid	338689 [184341.75, 708644.75]	279333 [181035, 377071]	3.97E-02	1.2	0.8	HMDB00755
	Pimelic acid	43185.5 [22537.75, 65656.5]	32909 [20846, 54552]	4.64E-02	1.1	0.8	HMDB00857
	Vanillic acid	29921 [17025.5, 57261.25]	21757 [13326, 42858]	1.47E-02	1.1	0.7	HMDB00484
Nucleotide	Cytidine	752.5 [285.5, 1778.75]	2356 [1046, 5837]	3.53E-05	2.3	3.1	HMDB00089
	Ratio of Uridine/Cytidine	10.83 [4.654, 29.632]	3.19 [1.075, 8.44]	6.34E-04	1.7	0.3	HMDB00296/HMDB00089
	Ratio of Hypoxanthine/Inosine	6.15 [3.033, 12.276]	9.81 [6.388, 15.86]	1.14E-02	1.2	1.6	HMDB00157/HMDB00195
	Ratio of Xanthine/Xanthosine	2.86 [1.745, 4.365]	3 [1.799, 6.264]	4.15E-02	1.1	1	HMDB00292/HMDB00299
Alcohols	Myoinositol	1730373.5 [994205.5, 2654704.25]	1286132 [810257, 1807007]	1.65E-02	1.1	0.7	HMDB00211
Lipids	O-Phosphoethanolamine	29426 [14883, 41505]	30719 [21563, 54094]	3.43E-02	1.2	1	HMDB00224
Vitamin	Pantothenic acid	53486.5 [36993.25, 83799.75]	91072 [63407, 120342]	1.94E-04	1.9	1.7	HMDB00210
Hormone	Normetanephrine	16594 [7148.75, 26553.25]	11305 [5759, 22922]	4.56E-02	1.2	0.7	HMDB00819
Indoles	Indoxyl sulfate	117741 [69175.75, 267423.25]	65358 [38260, 126046]	6.65E-03	1.2	0.6	HMDB00682

Note: Differential metabolites were selected according to VIP>1 and $P<0.05$. Values are expressed as medians (IQR). *P* values were calculated from t test for continuous variables and adjusted by FDR method; VIP, variable influence on projection; FC, fold change; HMDB, The Human Metabolome Database; T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy.

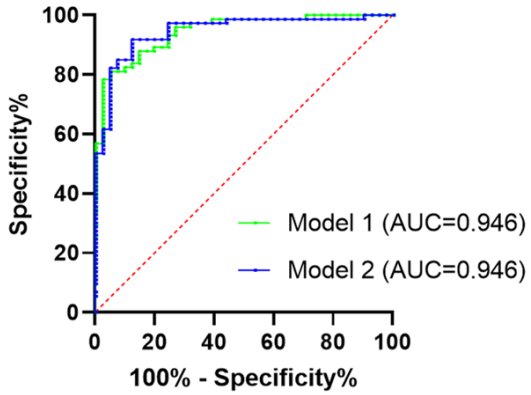

Figure S4. ROC curves of serum or urine candidate biomarkers for the prediction of DPN in T2DM patients. Model 1: Serum potential biomarkers; Model 2: Urine potential biomarkers. Serum biomarkers include β-alanine, ratio of β-alanine/L-aspartic acid, caproic acid, and ratio of L-arabinose/L-arabitol. Urine biomarkers include gluconic acid, erythritol, galactonic acid, guanidoacetic acid, cytidine, and aminoadipic acid. T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy; ROC, receiver operating characteristic; AUC, area under the curve.

Table S5. Performance of two models for the prediction of DPN in T2DM patients

Model	Features	Number of features	AUC	95% CI	*P* value
Model 1	Serum candidate biomarkers	4	0.946	0.908 to 0.984	<0.0001
Model 2	Urine candidate biomarkers	6	0.946	0.905 to 0.987	<0.0001

Note: The overall *P* value against a null model was calculated using the Wilcoxon signed-rank test. AUC, area under the curve. Model 1, serum biomarkers; Model 2, urine biomarkers. Serum biomarkers include β-alanine, ratio of β-alanine/L-aspartic acid, caproic acid, and ratio of L-arabinose/L-arabitol. Urine biomarkers include gluconic acid, erythritol, galactonic acid, guanidoacetic acid, cytidine, and aminoadipic acid. T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy.

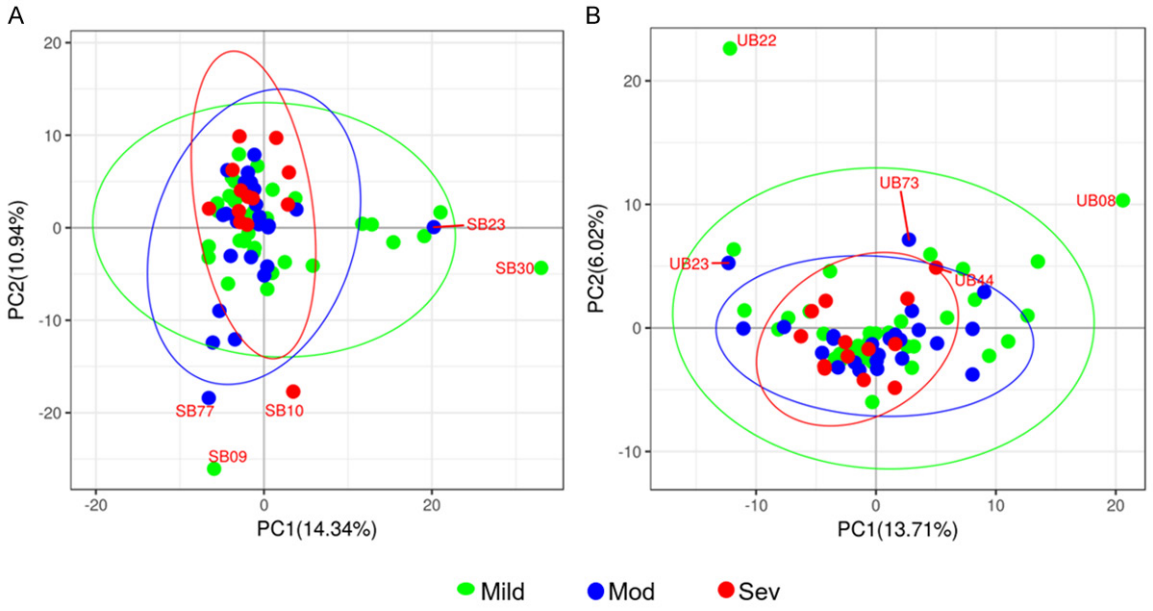

Figure S5. PCA score plot for metabolic profiling in different stages of DPN. A. PCA score plot of serum. B. PCA score plot of urine. Mod: moderate, Sev: severe. PCA, principal component analysis; DPN, diabetic peripheral neuropathy.

Table S6. Serum and urine metabolites that were significantly changed among DPN mild, moderate, and severe groups

Sample	Class	Name	Mild	Mod	Sev	Mod-Mild			Sev-Mild			Sev-Mod			HMDBID
						P	VIP	FC	*P*	VIP	FC	*P*	VIP	FC	
Serum	Amino Acids	β-Alanine	55879 [8632, 97491]	95148.5 [8370.5, 132486.5]	98098 [89561, 158390]	0.221	0.7	1.7	1.46E-02	1.8	1.8	0.17	1.2	1	HMDB00056
		Ratio of β-Alanine/L-Aspartic acid	0.36 [0.062, 0.696]	0.69 [0.052, 0.977]	0.87 [0.564, 1.281]	0.534	0.1	1.9	4.82E-02	1.4	2.4	0.179	1.2	1.3	HMDB00056/ HMDB00191
		L-Kynurenine	1334 [1150, 2105]	1216 [1091.25, 1588.5]	1406 [934, 1584]	4.12E-02	1.5	0.9	2.00E-02	1.6	1.1	0.399	0.8	1.2	HMDB00684
	Alkylamines	Hydroxyl-amine	10549 [8358, 11804]	9702.5 [6673.25, 13368.25]	12303 [10015, 15898]	0.498	0.2	0.9	4.12E-02	1.5	1.2	1.64E-02	2.1	1.3	HMDB03338
		Putrescine	15484 [12594, 18337]	19872.5 [13317.5, 36915.25]	13754 [9585, 19630]	4.47E-02	2.7	1.3	0.663	0.4	0.9	0.345	1.1	0.7	HMDB01414
Urine	Amino Acids	D-2-Hydroxy-glutaric acid	91810.5 [69438.5, 132088]	96541 [77142, 121917.5]	131198 [112830, 161394]	0.505	0.2	1.1	0.112	2	1.4	3.57E-02	2.9	1.4	HMDB00606
		Glycine	148989 [80609.75, 240285.5]	232547.5 [180082.5, 308024]	187539 [152594, 264639]	1.02E-02	1.9	1.6	0.386	1.4	1.3	0.319	0.8	0.8	HMDB00123
	Carbohydrates	L-Arabinose	1640506.5 [963609.75, 2320747.75]	1279853 [754335, 1633557.75]	2393576 [1502214, 4344509]	0.228	0.9	0.8	0.052	2.2	1.5	1.79E-02	2.8	1.9	HMDB00646
		D-Xylose	1976273.5 [1300310, 2562204.25]	2442736 [1817021.25, 2754439.75]	2740645 [2183104, 3063395]	0.099	1.3	1.2	1.71E-02	2	1.4	0.23	0.9	1.1	HMDB00098
	Organic Acids	Adipic acid	56479.5 [28866.25, 92073.25]	50358 [32586.5, 62677.5]	60931 [34814, 121230]	0.054	1.4	0.9	0.426	0.6	1.1	4.50E-02	2.6	1.2	HMDB00448
		cis-Aconitic acid	1280304.5 [904589.25, 1719659.5]	1877644 [1287011.75, 2177731.5]	2003043 [999083, 2294801]	2.19E-02	2.5	1.5	4.57E-02	2.3	1.6	0.583	0.4	1.1	HMDB00072

Note: Differential metabolites were selected according to VIP>1 and *P*<0.05. Values are expressed as medians (IQR). ANOVA test was conducted in the comparison among DPN different degrees. T-test was performed in the comparison between two groups. FC, fold change; HMDB, The Human Metabolome Database; T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy.

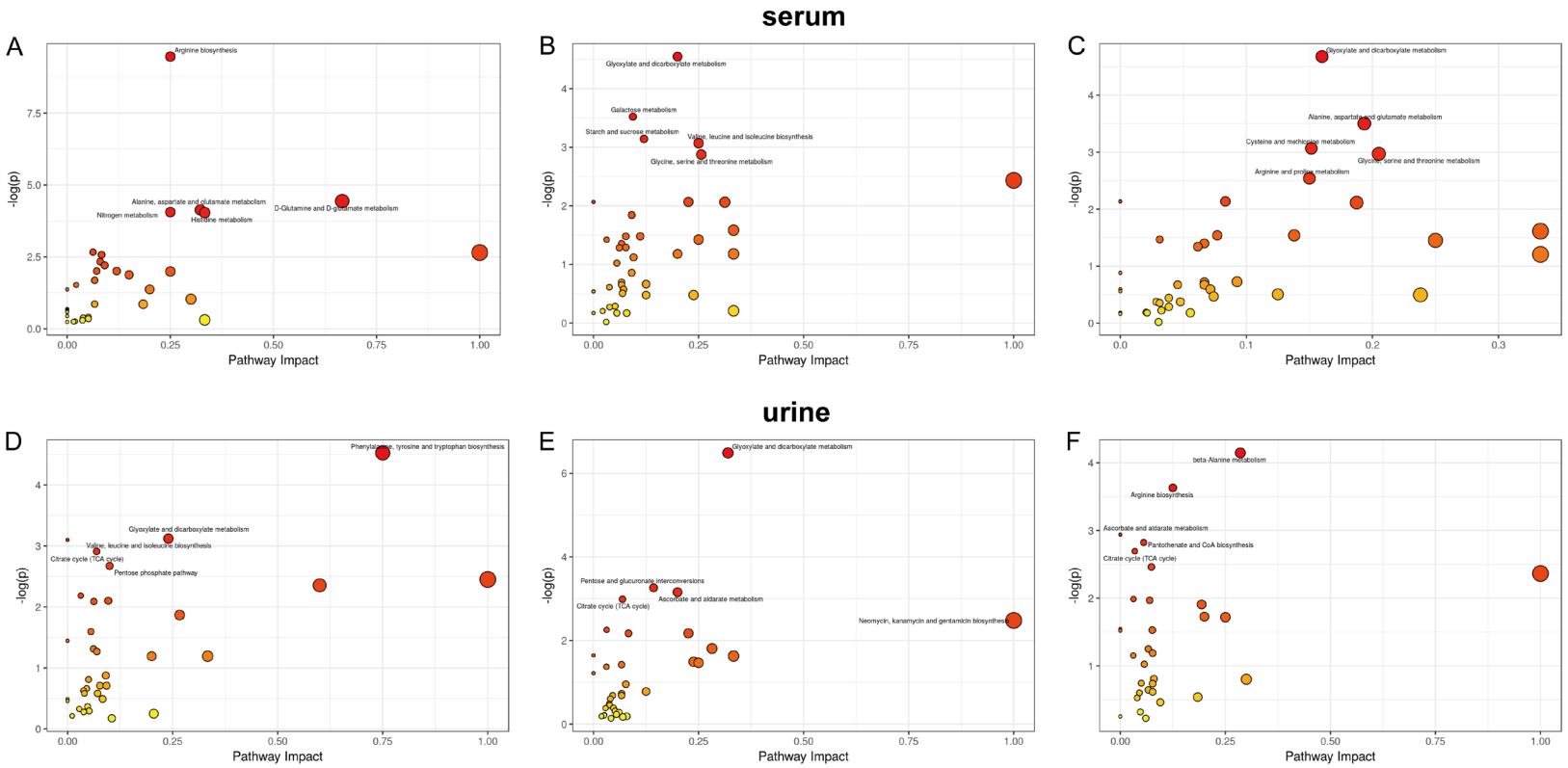

Figure S6. MPEA results of serum and urine key metabolic pathways. A. Overview of metabolic pathway analysis in serum (Mod vs Mild). B. Overview of metabolic pathway analysis in serum (Sev vs Mild). C. Overview of metabolic pathway analysis in serum (Sev vs Mod). D. Overview of metabolic pathway analysis in urine (Mod vs Mild). E. Overview of metabolic pathway analysis in urine (Sev vs Mild). F. Overview of metabolic pathway analysis in urine (Sev vs Mod). MPEA, metabolic pathway enrichment analysis; Mod, moderate; Sev, severe.